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Optimization of adenoviral vectors for cancer suicide gene therapy

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Ce mémoire intitulé :

Optimization of adenoviral vectors for cancer suicide gene therapy

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RESUMÉ

Les adénovirus en combinaison avec des gènes suicides sont parmi les modalités thérapeutiques les plus applicable pour le traitement du cancer. Un des gènes suicides couramment utilisés pour la thérapie génique du cancer est le HSV-TK. Dans le cadre de mon projet de recherche, nous avons cherché à améliorer l'approche thérapeutique du gène suicide HSV-TK par la coexpression de la guanylate kinase (GK). À cette fin, nous avons construit deux adénovirus : un co-exprimant la gène de la TK et le gène rapporteur GFP; et l'autre, co-exprimant le gène de la TK et celui de la GK; ces gènes étant contrôlés par le promoteur inductible CMV5(CuO). Nos résultats ont démontré que l'activité de la combinaison TK-GK était équivalente et non supérieure à celle de TK seule; et cela, dans différentes lignées de cellules cancéreuses comme le glioblastoma humain U87 et la lignée de tumeur ovarienne TOV21G. Nous avons de plus démontré qu'il n'en était pas différent avec des cellules normales (comprenant les cellules fibroblastes humaine MRC-5 et les cellules endothéliales de veine ombilicale humaine Huvec). Ainsi, nous avons conclu que dans les modèles étudiés, l'activité GK ne potentialise pas la cytotoxicité du gène TK.

J'ai aussi évalué différentes stratégies pour la construction de virus réplicatifs. Nous avons cherché la meilleure stratégie pour insérer un transgène (GFP) près de la région E4 de l'adénovirus, afin de conserver intact la région E1 qui est essentielle pour la réplication du virus. Le clonage de transgènes dans la région E4 affecte la réplication du virus et l'expression du transgène. J'ai démontré qu'une configuration où un promoteur inductible CR5 contrôle l'expression du transgène orienté vers l'ITR de gauche représente la meilleures option pour optimiser la production virale (i.e. son titre) et l'expression du transgène. Enfin, contrairement à un autre gène suicide étudié dans notre laboratoire, nous avons déterminé que la surexpression du gène TK, obtenue en augmentant la charge virale ou par réplication du virus, n'améliore pas l'activité cytotoxique dans les cellules U87. Ainsi, l'augmentation de l'activité du gène de la TK par la réplication, ou par la combinaison avec la GK n'a pas d'incidence sur l'activité cytotoxique; suggérant que l'activité de la TK n'est pas le facteur limitant.

Mots clés : vecteurs adénoviraux, thérapie génique du cancer, gènes suicide, pro-drogue, promoteur inductible, cumate

ABSTRACT

The adenoviruses in combination with the suicide genes are among the most applicable forthcoming therapies for cancer treatment. HSV-TK is one of the well-studied suicide genes that are currently tested for cancer treatment. In this project, we tried to improve the therapeutic effect of the suicide gene HSV-TK via its coexpression with guanylate kinase (GK). In order to achieve that, we constructed two adenoviruses: one coexpressing TK with the reporter gene (GFP), and the other, coexpressing TK and GK, which genes are controlled by CMV5(CuO) inducible promoter. Our results showed that the activity of the combination of TK-GK was equivalent to TK alone in different cancer cell lines including human glioblastoma (U87) and ovarian cell lines (TOV21G). We also demonstrated there were no difference in the activity of both viruses in normal cells as well (such as human fibroblast cells MRC-5 and human umbilical vein endothelial cells Huvec). In conclusion, we determined that in our platform, the activity of GK does not improve the cytotoxic effect of TK gene.

We also evaluated different strategies for construction of the replicative viruses. We looked for the best strategy to insert our transgene (GFP) in the vicinity of the E4 region of the adenovirus, in order to conserve the E1 region, which is essential for the replication of the adenovirus. In general the cloning of the transgene in the vicinity of the E4 region affects the replication of the adenovirus and the expression of the transgene. We demonstrated that the configuration in which the inducible promoter CR5 controls the expression of the transgene oriented toward the left ITR represents the best option for optimizing the viral production (i.e. the titer) and the transgene expression. Finally, in contrast to another suicide gene studied in our laboratory, we determined that the overexpression of TK gene in U87 cells, obtained from the increasing MOI or through replication, did not improve the cytotoxic activity nor the bystander effect. In conclusion, increasing the activity of the TK gene via replication or combination with another suicide gene (GK) has no incidence on cytotoxic activity; suggesting that the activity of TK is not a limited factor.

Key words : adenoviral vectors, cancer gene therapy, suicide genes, prodrug, inducible promoter,cumate

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LIST OF ABREVIATIONS

% : percentage
µg : microgram
µl : microliter
Δ : delta,deletion
AdV: adenovirus
BE : bystander effect
bp: base paires
CAR: Coxackie and adenovirus receptor
CD: cytosine deaminase
UPRT: uracil phosphorybosyl transferase
CMV5: human cytomegalovirus promoter
CMV5(CuO): cumate regulatory promoter (repressor configuration)
CR5 : cumate regulatory promoter (activator configuration)
cTA : transactivator of cumate operator
CuO: cumate operator
CymR : repressor of the cumate operator
DMEM : Dulbecco's Modified Eagle's Medium
E. coli : *Escherichia coli*
e.g.: example
GCV : ganciclovir
GDEPT: gene directed enzyme/ prodrug gene therapy
GFP: green fluorescent protein
GJIC: gap junction intracellular connection
GK : guanylate kinase
HSV-TK : herpes simplex virus TK gene
IC_{50}: 50% reduction in viability
IRES: internal ribosomal entry site
Kb : kilobases
KDa: kilodaltons
LTR: Long Terminal Repeat
milieu LB : Luria-Bertani madium
MLP (mlp) :major late promoter
MOI: multiplicity of infection
pAd : adenoviral plasmide
PCR : polymerase chain reaction
PFU: plaque forming unit
PS : protease
RCA : replication competent Adenovirus
RITR: right terminal repeat
RVs: retroviruses
$TCID_{50}$: Tissue Culture Infectious $Dose_{50\%}$
TK : thymidine kinase
TP: terminal protein
VP16 : transactivation domain of mammalian cells

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Chapter 1 :

Introduction

1.1 Gene therapy

Gene therapy, which emerged in the late 1980's to early 1990's, as part of modern molecular medicine holds great promise for the treatment of both acute and chronic diseases. It has proved by now its potential in a number of clinical trials aimed to develop a cure for several inherited and acquired genetic disease including various type of cancer, coronary artery disease, and cystic fibrosis (1;2). Gene therapy is essentially an approach that aims at correcting defective genes responsible for disease development, in which the delivered gene enters the cells and turns them into small factories that produce a therapeutic protein for a specific disease.

Despite the numerous encouraging results demonstrated by gene therapists so far, this technology is presently facing very serious obstacles associated with the development of the technical means to deliver corrective genes to the site of the disease in a safe and efficient manner. These obstacles include: poor delivery efficiency, high cost, time-consuming vector preparation, toxicity, immunogenicity, and oncogenicity, as well as short-term transgene expression and poor expression levels.

1.2 Gene delivery methods

Two distinct methods of delivering the therapeutic genes are possible. Gene transfer can be carried out '*in vivo*', where the vector containing the expression cassette is delivered directly to target cells within the individual. Alternatively, due to the inability of certain vectors to infect or reach the target tissue, cells are removed, grown *in vitro*, and infected/ transfected with the recombinant vector. The altered cells producing the foreign protein are then transplanted back into the patient, which is known as '*ex vivo*' gene therapy.

There are essentially two types of gene delivery vehicles, known as vectors including non-viral and viral vectors. In both cases, the gene of interest is first placed in

the vector, which is capable of delivering the gene into specific target cells for its expression. Non-viral methods make use of cationic lipids, polymers, targeting proteins, and calcium phosphate, as well as mechanical methods such as electroporation. Non-viral vectors were utilized as safer alternatives for gene delivery *in vitro* and *ex vivo* but their success for in vivo gene therapy has been limited due to low efficiency and safety issues. Viral methods make use of genetically engineered retroviruses, adenoviruses, adeno- associated viruses (AAVs), and other viruses that have been optimized for facilitated gene transfer procedures. However, the use of viral vectors was narrowed due to the safety concerns. There is no single best method for all applications.

There are certain stringent criteria for a candidate gene delivery system or "vector". First, the vector should be stable *in vivo* for a time period sufficient to achieve gene delivery. Second, administration of the vector should not be associated with a significant host immune response or toxicity.

1.2.1 Non- viral vectors

Non-viral gene delivery is an important approach in order to stablish safe *in vivo* gene therapy in clinic. The use of non-viral delivery methods has become routine for cell lines grown *in vitro* due to the simplicity and relatively high efficiency of gene transfer in a wide range of cell lines. However, due to some of the disadvantageous features, their application *in vivo* is limited. Non- viral vectors have a very low gene transfer efficiency compared to the viral vectors. Thus, except for the most recalcitrant cell lines, non-viral gene transfer has become the method of choice for cell lines grown in vitro. There are several options for non- viral gene delivery. The simplest method is the direct introduction of therapeutic DNA into target cells. This approach is limited in its application because it can be used only with certain tissues and requires large amount of DNA. Another non- viral approach involves creation of an artificial lipid sphere with an aqueous core. A wide variety of lipid molecules used as gene carriers has been reported and compared over the last twenty years. The liposomes, which carry the

therapeutic DNA, are capable of passing the DNA through the target cell membrane via the endocytotic process. Therapeutic DNA can also get inside target cells by chemically linking the DNA to a molecule that will bind to specific cell receptors. Once bound to these receptors, the therapeutic DNA constructs are engulfed by the cell membrane and passed into the interior of the target cell.

1.2.2 Viral vectors

Gene therapy has evolved due to the development of a number of biotechnology weapons including viral vectors that lead to a longer expression of the transgene. The viral vectors used as gene delivery vehicles include retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, vaccina viruses and more recently the lentiviruses. In contrast to non- viral vectors, viral vectors are generally more efficient in terms of percentage of cells transduced, are more complex and time-consuming to prepare, and require a greater degree of expertise in handling.

Today, the two most widely used viral vectors for gene therapy, are the retroviruses (RVs) and adenoviruses (AdVs). RVs are capable of integrating the host's genome and give a permanent expression of the recombinant gene. However, they are difficult to grow to high titres and purify for direct administration, which require the patient's cells be cultured and transduced *in vitro.* Finally, there is always the risk of insertional mutagenesis and/or activation of cellular oncogenes. Replication defective vectors derived from simple retroviruses or the most complex genomes of lentiviruses continue to offer the advantages of long term expression, cell and tissue specific tropism for the delivery of the therapeutic genes (3).

AdVs on the other hand, are not integrated into the host genome and shown transient expression in dividing cells but longterm expression in quiescent cells such as muscular cells. AdVs are one of the most studied viral vectors, which are considered as one of the best gene delivery vehicles developed so far.

1.3 Adenovirus Biology

1.3.1 Historical perspective

Adenoviruses have been first isolated and characterised in the early 1950's by Row et al, in an attempt for the study of the causal agent of acute respiratory infections. They observed the spontaneous degradation of primary cell cultures extracted from human adenoids. In 1954 Hilleman and Werner isolated agents from respiratory secretions that induced cytopathic changes in cultures of human cells while studying an epidemic of respiratory illness among army recruits. In 1956, the term adenovirus was designated for those viral agents, due to the original source of the tissue (adenoid) from which the virus was isolated. Adenoviruses are a frequent cause of acute upper respiratory tract (URT) infections. In addition, they also cause a number of other types of infections.

1.3.2 Classification

Adenoviruses belong to the family of Adenoviridae. This family is subdivided into two genera, Mastadenoviruses and Aviadenoviruses. Aviadenovirus genus is limited to viruses of birds, and Mastadenovirus genus includes a wide variety of viruses such as human, simian, bovine, equine, porcine, ovine, canine, and opossum viruses. To date, 47 human adenovirus serotypes have been isolated based on the type-specific neutralization by antisera which results predominantly from antibody binding to epitopes on the virion hexon protein and the terminal knob portion of the fiber protein (4). Each serotype was then classified into one of the six subgroups (see Table I), A-F, based on their ability to agglutinate red blood cells. Among all of the serotypes, group C viruses are the most studied in virology and molecular biology, which are non-tumorigenic and consist of AdV group C viruses serotypes 1, 2, 5 and 6. The serotypes 2 and 5 (AdV2 and AdV5) are most commonly used for gene delivery vector development.

Subgroup		Hemagglutination groups	Serotypes	Oncogenic potential	
				Tumors in animals	Transformation in tissue culture
A	IV	(little or no agglutination)	12,18,31	High	+
B	I	(complete agglutination of monkey erythrocytes)	3,7,11,14,16, 21,34,35	Moderate	+
C	III	(partial agglutination of rat erythrocytes)	1,2,5,6	Low or none	+
D	II	(complete agglutination of rat erythrocytes)	8,9,10,13, 15,17,19,20,22–30, 32,33,36–39,42–47	Low or none (mammary tumors)	+
E	III		4	Low or none	+
F	III		40,41	Unknown	

Table I: classification of human adenoviruses (Adapted from Shenk, T, 1996)

1.3.3 Morphology

All adenovirus particles consist of non-enveloped, icosahedral protein capsids with a diameter of 60-90 nm and the inner DNA/protein cores. 13% of the particle mass is DNA and the remaining 87% are proteins. The outer capsid is composed of 252 capsomers: 240 hexons and 12 penton base subunits. The hexon consists of a trimer of the major polypeptide II with a central pore. Polypeptides VI, VIII, IX and IIIa are known as the minor peptides and all play associated structural roles in the outer shell. Polypeptides VI, VIII and IX are associated with the hexon and seem to be involved in stabilising the Hexon capsomers. Polypeptide IIIa seems to function as a linker between the viral capsid and the core and ultimately in viral assembly. Five copies of polypeptide II are associated to form the penton base protein, which is found at each vertex of the icosahedral particle and act as a base for the fiber protein. Polypeptide IV forms the trimeric fiber protein, which projects from the penton base at each vertex of the icosahedron (5). The trimeric fiber proteins are responsible for recognition and binding to a 46-kDa transmembrane cellular protein known as coxsackie/adenovirus receptor (CAR), while the penton base recognizes the cellular integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) and helps in the internalization step (6). The core of the virion is composed of four known proteins and the viral genome. Polypeptides V, VII and mu are the first three proteins, which are in contact with the viral DNA. Polypeptide VII is known as a predominant

protein of the core with about 1000 copies present per virion, positively charged and extensively associated with viral DNA. The fourth protein, which is known by the name of terminal protein, is attached to the 5' ends of the viral DNA, by a phosphodiester bond. The terminal protein plays an important role in DNA replication and mediates attachment of the viral genome to the nuclear matrix (7). Viral protease, the cysteine protease L3/P23, is located in the internal cavity at about 10 copies per virion. The PS encoded in the late cassette L3 as a 23 KDa polypeptide and is essential during the maturation and assembly of the virus in the nucleus of infected cells as well as for the uncoating during entry into the host cell (8).

1.3.4 Genome organization

All adenovirus genomes are linear, non-segmented, double stranded DNA of ~36 Kbp long and are divided into 100 map units (mu). Each end of the viral genome has a 100- to 150- base pair (bp) repeated DNA sequence known as inverted terminal repeat (ITR). The left end contains the signal for encapsidation (packaging signal). Both of the ITRs and the packaging signal are the cis-acting elements, which are necessary for viral DNA replication and packaging (9).

The viral genome is composed of five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and Iva2) and one late unit, which generates five families of late mRNAs (L1 to L5) (see Figure1).

The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and of a few cellular genes. E1A is the first gene to be transcribed directly after infection. Transcription of the E1A unit is controlled by a constitutively active promoter that includes a duplicated enhancer element. This gene codes for two different components sharing substantial sequences that are termed as 289R or (13S) and 243R or (12S) based on the number of amino acid residues. Those proteins are trans-acting transcriptional regulatory factors, which are necessary for transcriptional activation of early genes. Moreover the E1A proteins are primarily

concerned with modulating cellular metabolism to make the cell more susceptible to virus replication by inducing the host cell to enter the S phase of the cell cycle (10). The E1B gene codes for two different proteins known as E1B19KDa and E1B55KDa. Like E1A proteins, the E1B proteins also modulate cell cycle progression via targeting the cellular p53 tumour suppressor protein, which regulates progression from G1 to S. The E1B-55KDa protein binds to p53 within infected cells and it can block transcriptional activation by p53. The E1B19KDa gene product is analogous to the cellular Bcl-2 gene and is concerned with prolonging cell survival via an interaction with the Bax family (whose transcription can be promoted by p53) (11). The E1B-55 KDa protein also complexes with the ORF6 protein from the E4 region to modulate expression of the viral late genes.

Expression of the E2 region (E2A and E2B) leads to the synthesis of the DNA polymerase, pre-terminal protein and DNA binding proteins needed for viral DNA replication. The E3 genes, which are dispensable for the replication of the virus, code for proteins involved in counteracting the immune system after an adenoviral infection. The E3 gp 19KDa protein binds to the MHC class I heavy chain and prevents its transport to the cell surface, thereby inhibiting the differentiation of cytotoxic T lymphocytes directed against viral antigens (12). The E3-RID complex containing RIDα and RIDβ, a membrane protein, inhibits Fas induced apoptosis by internalizing Fas from the cell surface into lysosomes where Fas is degraded. The E3-14.7 KDa, a non-membrane protein, is involved in inhibition of TNF-induced apoptosis by interacting with cellular proteins in TNFR1- caspase pathway. The E4 transcription unit of Ad is located between map units 91.3 to 99.1 at the right hand end of the Ad genome and is transcribed in the leftward direction and is controled by the E4 promoter. The gene products derived from the E4 cassette termed ORF1, ORF2, ORF3, ORF3/4, ORF4, ORF6 and ORF6/7. They are mainly involved in viral DNA replication, late viral protein synthesis, shut off of host protein synthesis, production of progeny virions and preventing concatemer formation of viral genomes. Ad mutants that lack the entire E4 region or that fail to express ORF6 and ORF3 proteins are severely restricted for growth. These mutants show a complex phenotype, which includes a defect in virus particle assembly, failure to accumulate

normal nuclear and cytoplasmic levels of late messages and late protein synthesis, and failure to shut off host macromolecular synthesis at late times (13). It has been shown that the viruses that contain mutations in both ORF3 and ORF6 are as defective as the E4-deleted viruses. Other studies have shown that ORF3 and ORF6 can partially compensate for each other's defects and are involved in enhancing of late gene expression through nuclear stabilization of late mRNAs and indirectly increase the efficiency of viral DNA synthesis (14). The product of the late gene is expressed after processing a 20-Kbp primary transcript driven by the major late promoter (MLP). This large transcription unit is then processed by differential poly (A) site and splicing in order to generate 18 mRNAs. Those mRNAs are grouped into five families called L1-L5 all of which are responsible of producing the viral capsid proteins (9).

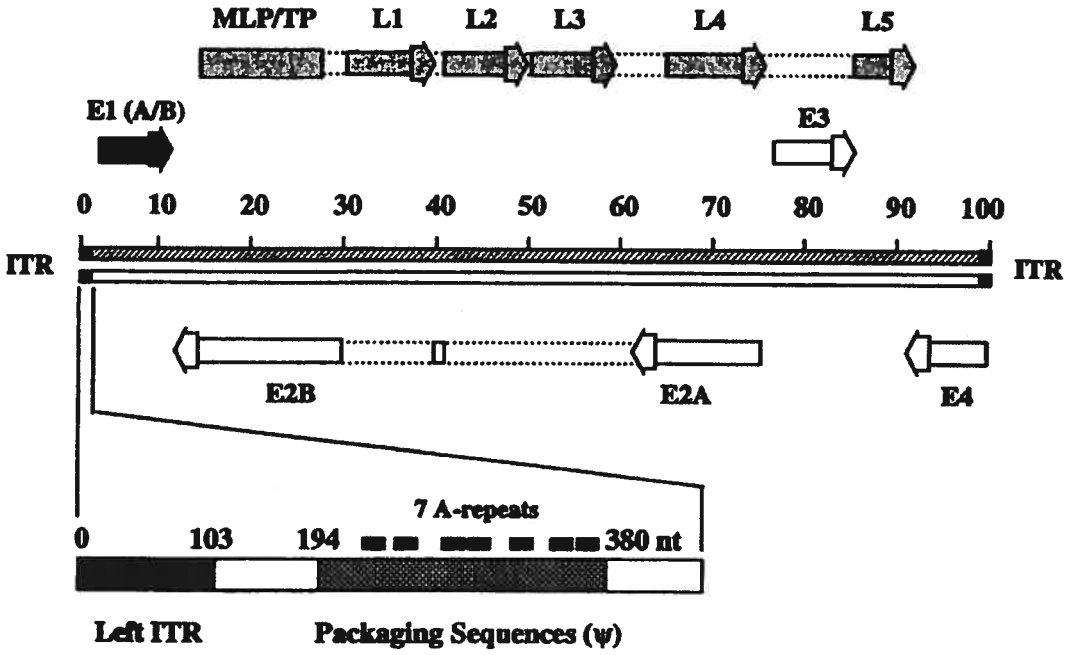


Figure 1: AdV_5 genome structure and essential cis elements. The length of the AdV5 is ~36 Kbp, divided into 100 mu. The upper strand is the r-strand and the lower strand is the l-strand. Black squares at the ends of both strands represent ITRs. The arrows represent different transcription units. The empty box on top represents the location of the MLP and tripeptite leader sequences. The left cis elements, including the ITR and packaging signal (Ψ), are enlarged; in addition, the positions of the A-repeat motifs of the packaging sequences are shown (9).

1.3.5 Replication cycle

AdV is typical of lytic DNA viruses. The replicative cycle of Ad starts from entering cells and ends at release from the lyzed, infected cells. The terminal globular domain or "knob" region of the homotrimeric fiber of the Ad capsid is responsible for the primary virus attachment to the cellular receptor, the coxsackie and adenovirus receptor (CAR) (15). Following the initial attachment, the interaction between an RGD-motif of the penton base protein with a cell surface integrin molecule ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$), serving as a secondary or internalization receptor, triggers the virus uptake by clathrin-dependent receptor-mediated endocytosis (16). The endosomal uptake of the virus and release into the cytoplasm is accompanied by a stepwise dismanteling of the capsid, leading to the microtubule assisted transport and finally very efficient delivery of the core protein-coated genome to the nucleus. These may involve interactions with surface receptos, exposure to low pH in endosomes or association with intracellular factors. It has been shown that for capside dissociation and genome release the structural protein VI has to be degraded by the viral L3/P23 protease (17). The transport of viral DNA to the nucleus consists of the binding of the terminal protein to the nuclear matrix and entry of the genome into the nucleus in association with one or more NLS- containing proteins. There are three steps for the replication cycle, which are the early transcritional phase, DNA replication phase and late transcriptional phase. Three goals are accomplished at the first phase of the early transcriptional phase: firstly, to create an optimal environment for viral replication by expression of the E1A and E1B gene products which play a role in the induction of the host cell to enter the S phase of the cell cycle: secondly, to protect the infected cells from the antiviral defense system of the host by expressing the products of E3 gene: thirdly, to express the viral gene products that are essential for viral DNA replication.

During DNA replication phase, four virus- encoded proteins are known to be involved. TP, a 55 KDa terminal protein, that acts as a primer for initiation of synthesis, AdDBP, that is a DNA binding protein, and AdDNA POL, which is a 140kDa DNA-dependent polymerase. A cysteine protease (L3/P23) plays an important role in

proteolytic trimming of TP. The special TP/ITR structure serves as the origin of the viral DNA replication. Chain elongation is completed by two virus E2- coded proteins, the polymerase and single stranded DNA binding protein and cellular proteins (factor I and II).

The third phase of the replication cycle is the late transcription, which is primarily driven by the major late promoter (MLP). The late transcripts (L1-L5) derived from the MLP promoter possess a 5' tripartite leader sequence, which make them preferred over the cellular mRNA for translation.

1.3.6 Viral particle assembly

Assembly of the Ad virion generally begins in the cytoplasm when individual monomers form into hexon and penton capsomers. After their production, hexon and penton capsomers accumulate in the nucleus where the final assembly of the virion occurs. The intranuclear virion assembly starts about 8h after infection and leads to the production of 10^4 to 10^5 progeny particles per cell, which can be released after final proteolytic maturation by cell lysis 30- 40 h post infection completing the viral life cycle. There are multiple steps along the assembly process: (a) formation of the major structural unit of capsid (capsomers of hexon and penton), (b) assembly of empty capsids, (c) insertion of viral DNA into capsids, and (d) proteolytic cleavage of maturation. The cis- acting packaging domain of AdV5 is located in the left end of the viral genome (194- 380 bp) as seven AT rich motifs. Deletion or manipulation of the A-repeats of the packaging signal result in attenuation or loss of packaging of the viral genome (7). The cystein protease is essential for the maturation and assembly of viral proteis, and for the release of virions from infected cells (8).At the end of the lytic cycle, an E3- encoded protein (E3-11.6 KDa), called adenoviral death protein (ADP), allows for efficient release of viral particles by cell lysis (18).

1.4 Adenovirus vectors

Adenovirus vectors have become a very popular tool for gene transfer into mammalian cells (19). They are derived mainly from the AdV2 and AdV5 serotypes, because they are well characterized and by contrast to some other serotypes they are neither associated with severe illness nor tumorigenic in rodents. Adenoviral vectors are presently considered to be among the most effective viral vectors, based on the following properties: a) Ad vectors have a middle size of genome (~ 36 Kbp) that are suitable for the development of large capacity vectors and are stable through successive replication cycles; b) they are easy to generate and manipulate; c) they show a transducing efficiency of almost 100% in a variety of animal culture cells; d) They are quite stable and can be obtained in high titres (e.g., 10^{11}-10^{12} plaque forming units (PFU)/ml); e) They are capable of infecting a broad host range in vitro and in vivo (effective in dividing and non-dividing or terminally differentiated cells); f) no significant side effects have been reported following early clinical application or vaccination with live replicative AdVs; g) Their genome rarely integrates into the host chromosome, which is suitable for applications requiring transient gene expression (20). However, the hybrid adenovirus/ retrovirus or adenovirus/ adeno-associated virus vectors can be used to achieve integration of transgenes into the genome and long- term gene expression (21;22).

Two types of adenoviral vectors have been used for gene therapy and vaccination purposes including replication competent and replication defective adenoviruses. Replication competent AdVs are ideally suited for applications in which the high level transient expression of transgene is required without the need for the stability of expression, such as in the case of vaccination. On the other hand, replication-defective adenoviruses are mainly used for protein production and functional studies and gene therapy purposes. The encapsidation capacity of adenovirus is estimated to be up to 105% of the genome, 2Kbp can be tolerated without deletion (23). The deletion of the dispensable E3 region (2.7 Kbp) increase the Ad packaging capacity allowing for the insertion of up to 4.7 Kbp of foreign DNA into the replication- competent AdV.

Three classes of the defective adenoviral vectors are currently being developed for gene therapy purposes (see Figure 2). First generation adenovirus vectors are made by substituting an expression cassette for the E1 and/ or E3 region. Since the E1 products are necessary for viral growth, they must be provided in trans in specific cell lines such as 293 cells, which constitutively express the E1A and E1B genes. Various deletions in E1 region can accommodate up to 3.2 Kbp and the deletions in E3 region up to 3.1 Kbp of the foreign DNA. Since adenovirus can package approximately 38 Kb without affecting growth rate and viral titer (24), E1/E3 deleted viruses allow the cloning of about 8.2 Kbp. The main advantage of the first generation AdVs is that they are easier to construct and produce than the other classes of defective adenoviral vectors. One problem for their production is the appearance of replication competent AdV (RCA) during multiple passages in 293A cells. The main disadvantage of the first generation adenovirus vectors is that they elicit a significant immune response in vivo, mainly due to the synthesis of viral proteins and results in a loss of therapeutic gene expression 1 to 2 weeks post infection (25;26). Therefore, additional genes necessary for viral DNA replication have been deleted, resulting in the second generation of adenovirus vectors. Different cell lines have been constructed that express the E2a DNA binding protein, the E2b-encoded terminal protein and viral DNA polymerase, or all or most of the E4 products (27-29). The corresponding deletions should allow a maximum cloning capacity of 14 Kbp, and show reduced antigenicity in vivo, since they are less immunogenic. However, the duration of gene expression appears to be reduced. Third generation AdV is also represented by 'gutted' or 'helper-dependent' adenoviruses. These vectors are deleted for all the genes but only remain intact for the cis-acting sequences including the inverted terminal repeats and the packaging sequences, which are necessary for viral DNA replication and packaging. Since these gutless vectors are unable to replicate on their own, the presence of a helper virus (first generation AdV) and the appropriate complementing cells for propagation followed by careful purification are required. In theory such viruses can accomodate the cloning capacity up to 37 Kbp, however the disadvantage of this approach is due to the difficulty in separating the gutted vector from the helper virus. A further development involved the use of the Cre-

lox helper-dependent system, which allowed the production of helper-dependent AdV reasonably free of helper virus (30).

According to their current limitations, adenoviral vectors are not considered as the best candidates for treating genetic disorders, which need long- term expression of the therapeutic genes. On the other hand, they may be better candidates in treating diseases for which only transient gene expression is required such as cancer, cardiovascular and restenosis.

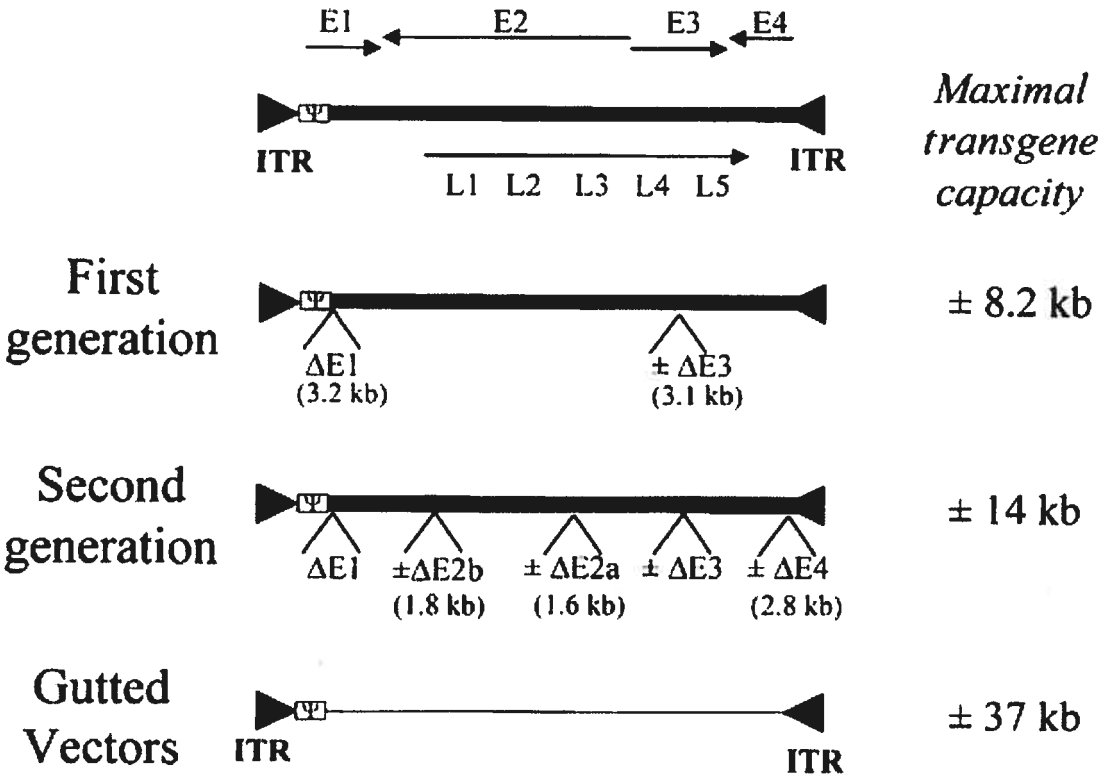


Figure 2. Genome structure of first generation, second generation and gutted adenovirus vectors. The location of the early and late transcription units (arrows) in the adenovirus genome (black bar) are shown on top of the figure. The ITRs are symbolized by triangles. The packaging signal (ψ) is highlighted by an open box. Non-adenoviral sequences in gutless vectors are represented by a thin line. The sizes of the largest deletions are indicated for each region, and the maximal insert sizes are indicated for each type of vector (30).

1.4.1 A new replication- competent defective AdV vector

In order to reach the short term high level expression of the transgene a new replication- competent defective AdV vector has been designed lacking the PS gene. As previously described the adenoviral PS is one of the essential late viral genes involved in many steps of the virus cycle. The PS is essential for the maturation and assembly of viral proteins and for release of virions from infected cells. By deleting an essential gene that is not involved in the early steps of viral replication, it becomes possible to obtain a localized replication without risk of viral shedding and thus obtain a greatly enhanced expression of the transgene. The PS deleted virus can be propagated in 293A-derived cell lines engineered to express PS (293A-PS) (see Figure 3). Such vectors are suitable for applications in which over-expression of the transgene such as in the case of vaccination, in situ therapy for tumors, protein production or the large scale production of other viral vectors such as adeno-associated virus (AAV).

It has been shown in another application that using a PS-deleted genome and the ectopic expression of PS gene via transfer vector allowed for positive selection of recombinant AdVs (31). With this method, since the PS-deleted mutant can not go beyond one round of replication in E1 expressing 293A cells expressing, only those recombinant genomes having the PS gene can lead to productive infection.

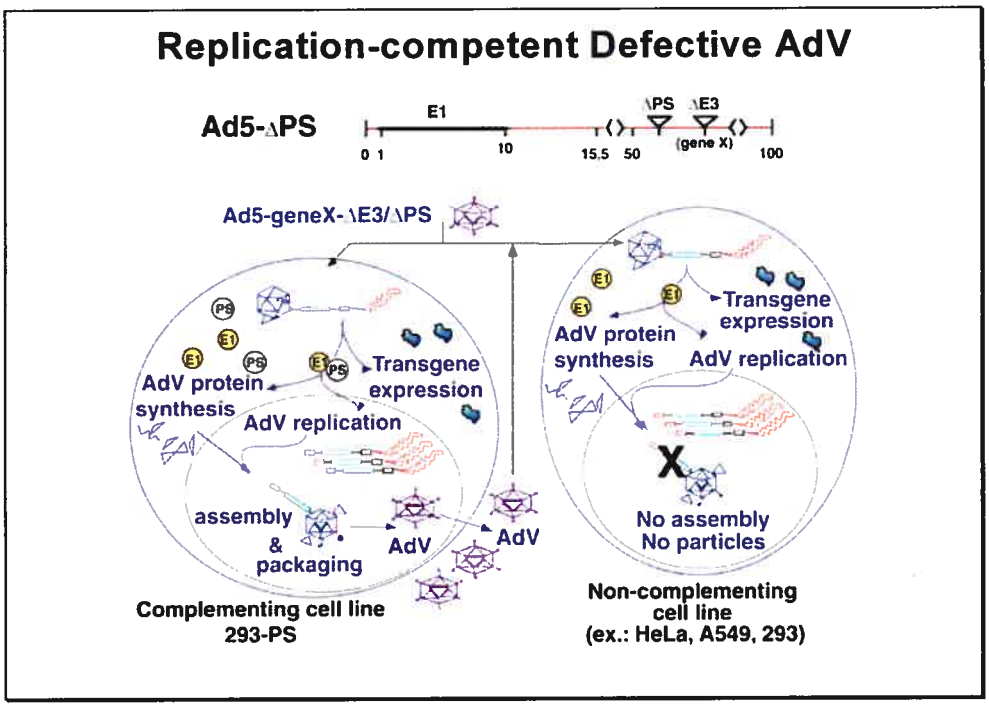


Figure 3: Replication- competent defective adenoviruses (single- round replication AdVs). Vectors deleted for PS gene can be completely replicated in complementary cell lines. In non-complementary cells, the DNA replicates, and because of the absence of the PS gene assembly and release of infectious virions could not occur (8).

1.4.2 Regulatable adenovirus vectors

A rigourous control of gene expression in human gene therapy strategies is important for both therapeutic and safety reasons. Inducible gene expression is required to regulate the expression of the proteins that are either cytotoxic or which interfere with Ad replication. Inducible expression cassettes using a chimeric promoter can be successfully used for some particular applications in which regulating transgene expression in vivo improves its safety and/or efficacy.

Since the bacterial tetracycline- regulated system for inducible expression in mammalian cells (32) is demonstrated as a suitable model, several systems based on bacterial regulatory elements have been successfully used in AdVs. A number of

inducible systems either endogenous to mammalian cells or using prokaryotic and insect regulatory systems are available. In our laboratory a new bacterial operon has been adapted to regulate gene expression in mammalian cells (33). In *Pseudomonas putida F1*, the degradative pathway for *p-cymene* to its benzoate derivative *p-cumate* consists of 6 genes organized in an operon (cym) (34). The cym operon is followed by the cmt operon that is responsible for the future degradation of cumate. The expression of the genes in both operons is regulated by a 28 kDa repressor molecule (CymR) that binds to the operator sequences downstream of the start site of the promoter. CymR is in a DNA-binding configuration only in the absence of effector molecules including cymene or cumate. We have used CymR to control gene expression in mammalian cells with two different strategies as shown in Figure 4 A and B.

The first strategy is based on using of CymR as a repressor that reversibly blocks the expression from the strong promoter. The Cumate operator is placed between the TATA box and the initiation site (19 or 40 bases from the TATA) and is able to mediate repression by the repressor very effectively. It is possible to regulate DNA binding of the repressor protein (CymR) from the operon sites. Therefore the expression of the transgene can be suppressed in the absence of the inducer cumate and induced in the presence of cumate (A). The second strategy involves in generating a chimeric cumate transactivator (cTA) by fusing of the bacterial repressor (CymR) to an activation domain of mammalian cells (VP16). Scince the DNA-binding ability of CymR is regulated by cumate binding, it is possible to regulate DNA binding of the fusion protein (CymRVP16) from the operon sites placed downstream of the minimal promoter (TATA box). Therefore the transactivation by the transactivator can be suppressed in the presence of the inducer cumate and induced in the absence of cumate (B).

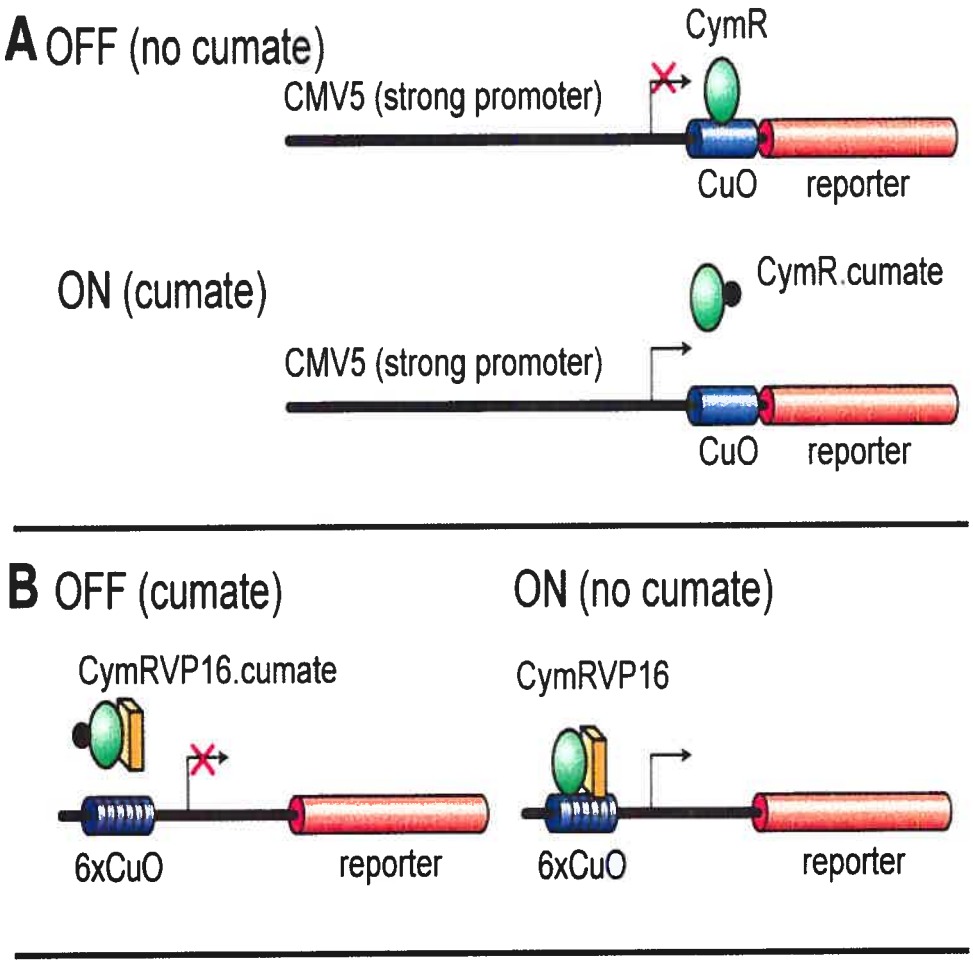


Figure 4: Controling the expression of the transgene via two different strategies of cumate switch system. A) Repressor configuration, B) Activation configuration (33).

1.5 Application of adenoviral vectors in gene therapy for cancer

The explanation of the molecular mechanisms underlying neoplastic transformation has resulted in understanding that cancer is a genetic disease originating from the accumulation of a series of acquired genetic lesions. Despite advances in the traditional treatments of cancer including chemotherapy, radiotherapy and reductive surgery, cancer remains one of the top causes of death in adult and children. In cases where the nature of the tumour is more difficult to treat (e.g., lung and brain cancers) or where diagnosis is not possible until very late (e.g., ovarian and pancreatic cancers), the traditional therapies have proven to be ineffective. Furthermore, traditional treatments have been videly associated with undesired side effects. It is apparent that novel therapeutic approaches will be required to obtain significant increases in clinical survival.

Gene therapy for cancer is a novel approach with the potential to selectively eradicate tumour cells while sparing normal cells from damage. There are several elements of concern regarding the safety and efficacy for gene therapy of cancer, which are: a) the target tissue; b) the transgene and its delivery and c) the host response. Among these factors, gene delivery is a key element. The requirements of an ideal gene delivery vector for cancer treatment should be evaluated based on the following criteria: a) high gene-transfer efficacy; b) large gene carrying capacity; c) regulation of gene expression; d) controlled cytotoxicity; e) targeted immunogenicity; f) ease of construction and manipulation; g) cost-effective production; h) safe and convenient application.

The existing approaches to gene therapy of cancer can be divided into five broad categories: (1) mutation compensation, (2) molecular chemotherapy, (3) genetic immunopotentiation, (4) genetic modulation of resistance/ sensitivity, and (5) oncolytic therapy or virotherapy.

The application of Ad for cancer therapy can be dated back to the 1950's, when wild type adenovirus was used to treat cervical cancer. Ad vector-mediated delivery of

tumor-suppressor genes, antisense oncogenes, suicide/toxin genes, and other effective genes have been successfully demonstrated to be effective in inducing tumoricidal effects and anticancer immunity in different animal models (35;36).

1.5.1 Molecular chemotherapy

A number of well- defined approaches to molecular chemotherapy for cancer have been developed. These include the administration of (1) toxin genes to eliminate tumour cells and the stromal cells that support them, (2) drug resistance genes to protect the bone marrow from myelosuppression induced by chemotherapy, and (3) genes that enhance the effect of traditional anticancer treatment. Traditional and molecular chemotherapy follow the same principle, where the pharmacological agents are employed. However, in the traditional chemotherapy, the toxicity of the drug is often displayed both in malignant and non- malignant cells. Molecular chemotherapy is designed to selectively target toxin expression to cancer cells in order to reduce the potential for non-specific cytotoxicity. In the latest approach, a non- toxic prodrug is administered and could be transformed into a toxic metabolite only in genetically modified cells, that ultimately leads to cell death (37).

1.5.1.1 Gene Directed Enzyme/ Prodrug Therapy of Cancer

One of the main goals in anti-tumour therapies is to target toxic agents in a specific and selective manner to tumour cells, while avoiding the normal cells from damage. This could be achieved by gene therapy that can specifically perform gene delivery to the target cells with the specific gene expression in the target cells. The gene directed enzyme/ prodrug therapy (GDEPT) is basically focused on the choice of therapeutic genes for cancer gene therapy, in particular on molecular chemotherapy or "suicide" gene therapy. GDEPT is defined as a two step therapeutic approach for cancer gene therapy. In the first step the transgene is delivered into the tumor and expressed using potential vectors including adenoviruses and retroviruses. The delivered transgene

encodedes for an enzyme, which is not toxic by itself. In the second step a non-toxic compound (prodrug) is administered and is selectively activated and converted to a potent cytotoxin by the expressed enzyme (38) (see Figure 5). In the choice of the appropriate enzyme/ prodrug combination, priority should be given to the enzyme. For ease of possible protein modification, the enzyme should be monomeric, of low molecular weight and with no requirement of glycosylation. It should have high catalytic activity under physiological conditions, fast and efficient prodrug activation even at low concentrations of the substrate, without dependence on further catalysis by other enzymes. Expression of the enzyme itself should not lead to cytotoxic effects because the required bystander effect would not be achieved if the cells were killed by the action of the enzyme alone. Several suicide gene/ prodrug combinations are currently under investigation, including cytosine deaminase (CD)/5-fluorocytosine (39), thymidine phosphorylase/5'-deoxy-5-fluorouridine (TP/5'-DFUR) (40), purine nucleoside phosphorylase (PNP)/6- methylpurine-2'-deoxyriboside (MeP-dR) (41) and herpes simplex virus thymidine kinase type 1 (HSV-TK) in combination with the prodrug GCV (42).

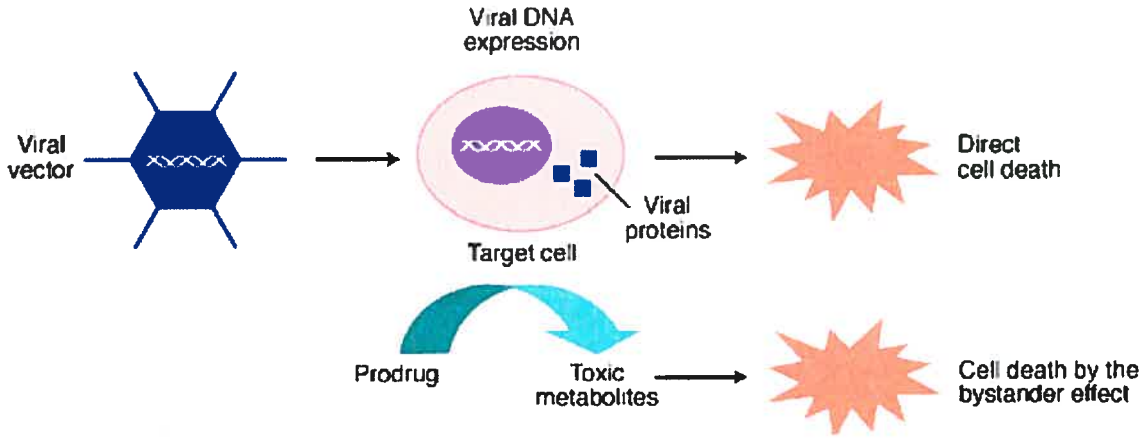


Figure 5: Schema of the Gene Directed Enzyme/ Prodrug Therapy via viral vectors.

1.6 Suicide gene therapy mediated by HSV-TK/ GCV system

To date, HSV-TK with the nucleoside analogue GCV is known as the most well- studied enzyme/ prodrug strategy in cancer. Frederic Moolten was the first to describe the potential of the HSV-TK/GCV system as a prodrug- activating gene therapy. It was shown that the transfection of tumor cells with HSV-TK gene makes these cells sensitive to GCV, a guanosine analog (42). In fact, in 1990 the initial studies of HSVTK/GCV gene therapy using retrovirus vectors showed that the expression of HSV-TK with GCV treatment generated enough phosphorylated GCV to inhibit mammalian DNA polymerase.

1.6.1 Characterization of the HSV-TK as compared to the mammalian TK

Mammalian thymidine kinase (TK) is a key enzyme in the pyrimidine salvage pathway phosphorylating to thymidine (dT) to thymidine monophosphate (dTMP) in the presense of Mg^{+2} and ATP. In the cell, dTMP is subsequently diphosphorylated and triphosphorylated and finally used as a DNA building block. Moreover, mammalian TK accepts only pyrimidines, whereas viral TK from Herpes simplex virus type 1 also accepts purines, and even the acyclic ribose analogue such as prodrugs acyclovir (ACV) and gancyclovir (GCV). In virus- infected cells those prodrugs are activated to their triphosphates and incorporated into the viral DNA resulting in chain termination. Thymidine kinase belongs to the α/β protein family. The core of the protein contains a parallel 5-stranded β- sheet and the sheet is surrounded by 12 α- helices covering in both side (43).

1.6.2 Characterization of the human Guanylate Kinase (GK)

Guanylate kinase catalyzes the phosphorylation of either GMP to GDP or dGMP to dGDP and is an essential enzyme in nucleotide metabolism pathway. In addition to being critical enzyme in the biosynthesis of GTP and dGTP, guanylate kinase

functions in the recovery of cGMP and is therefore, thought to regulate the supply of guanine nucleotides to signal transduction pathway components (44;45). Guanylate kinase activity has been observed in a variety of tissues and has been purified from many eukaryotic sources including bovine retina, bakers' yeast, hog brain, rat liver, and human erythrocytes.

Like the other enzymes involved in nucleotide metabolism, guanylate kinase is a target for cancer chemotherapy and is inhibited by the potent antitumour drug, 6-thioguanine (46). However, guanylate kinase activity is also required for antiviral drug activity in virus- infected cells. Activation of the anti-herpes guanosine nucleoside analogs acyclovir and ganciclovir, after an initial phosphorylation step by the viral thymidine kinase, is carried out by guanylate kinase. Therefore guanylate kinase is a key enzyme for cancer chemotherapy and antiviral drug activation in humans.

1.6.3 Mechanism of the HSV-TK/GCV system

GCV, an acyclic analog of the natural nucleoside 2' deoxyguanosine, is an antiviral agent widely used against human cytomegalovirus, herpes simplex virus type 1 and 2, varicella zoster and Epstein-Barr virus. GCV can be converted to its monophosphate form efficiently by thymidine kinase from HSV-1 (see Figure 6A). Subsequent reactions catalysed by cellular enzymes including guanylate kinase (monophosphate to diphosphate) and a number of nonspecific nucleoside diphosphokinases (diphosphate to trophosphate) (see Figure 6B). GCV- triphosphate competes with deoxyguanosine triphosphate for incorporation into elongating DNA during cell division, causing inhibition of the DNA polymerase and single strand breaks. These characteristics make the HSV-TK/ GCV combination particularly suitable for the eradication of rapidly dividing tumour cells.

In the last 15 years, more than 400 papers have discussed the potentiality of HSV-TK/ GCV for cancer GDEPT. Pre-clinical studies using adeno- and retroviral

vectors were performed in many different animal models and successful results were reported for established rodent glioblastomas (47;48), rodent liver metastases (49), human head and neck carcinomas (50), human mesotheliomas (51), murine hepatocellular carcinomas (52) and other tumour types.

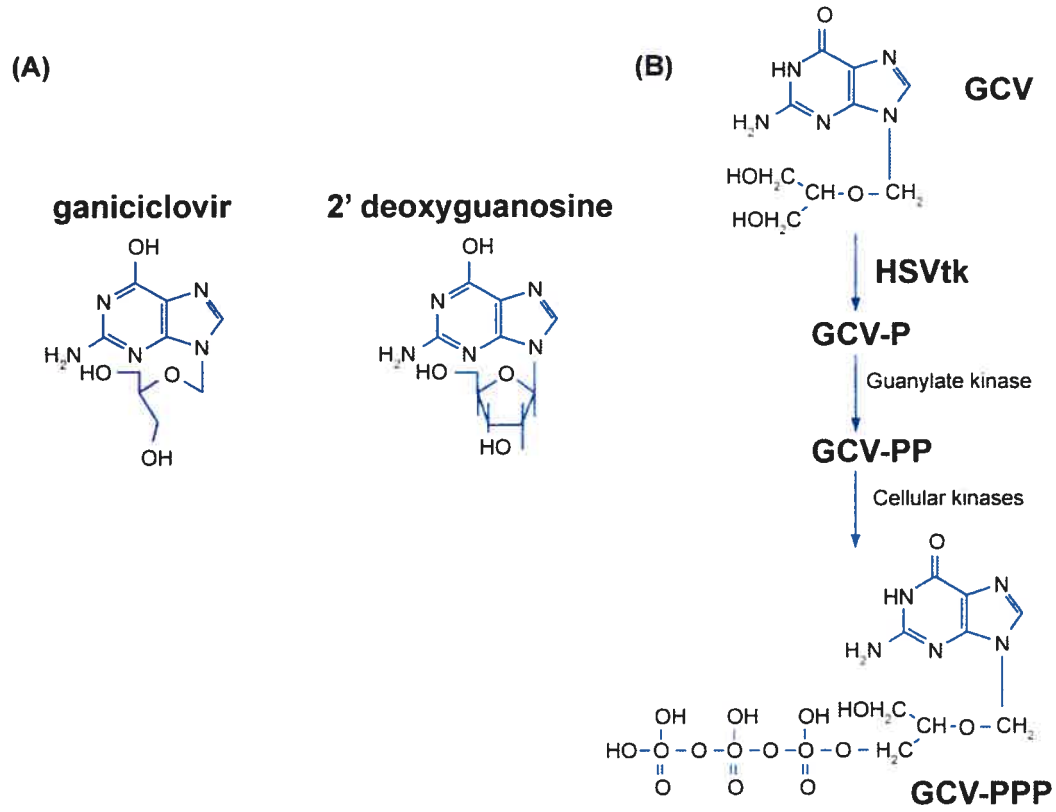


Figure 6: A) Comparison between ganciclovir (GCV) and nucleoside 2' deoxyguanosine. B) Metabolic pathway of the prodrug GCV. GCV is specifically phosphorylated by the herpes simplex virus 1 thymidine kinase (HSV-TK) to its monophosphate. Subsequently, GCV-monophosphate is converted to the diphosphate and triphosphate forms by guanylate kinase and other cellular enzymes and can be incorporated into elongating DNA, causing inhibition of the DNA replication and single strand breaks (adapted from Fillat et al., 2003).

1.6.3.1 Mechanisms of HSV-TK/GCV mediated cell killing

The mechanisms of HSV-TK/GCV induced cell killing is not completely understood. There are much evidence showing that both apoptotic and non- apoptotic pathways have been involved in HSV-TK/GCV mediated tumour killing pathway depending on the specific cell type (53;54). It has been shown that the apoptotic pathway is involved in inhibition of bcl-2 family (55). It has been shown that the manipulation of the intracellular levels of bcl-2 has been used to divert the mechanism of tumour cell death from apoptotic to predominantly non-appoptotic. By taking advantage of this fact scientists observed that the non-apoptotic cell killing pathway is associated with induction of heat shock protein (hsp70) expression *in vitro* and *in vivo* and also is involved in increasing the immunogenicity of tumour cells (56).

Several authors have investigated the different HSVtk/GCV- mediated tumour killing pathways depending on the specific cell type. It has been shown that the mechanism of cell death in HSV-TK expressing B16F10 murine melanoma cells is due to the irreversible cell cycle arrest at G2-M checkpoint and is independent of apoptosis (57). However, it has been shown that in human hepatocarcinoma cells the HSV/GCV induces cell death through apoptosis (58). In this case, the level of apoptosis was dependent on the p53 status of the cell being reduced in p53 negative cells compared to p53 positive cells. In addition, the up-regulation of Fas L was observed in both p53 positive and negative cells but the up-regulation of Fas was observed only in p53 positive cells. In contrast, it has been recently shown that in a model system of CHO cells, stably transfected with HSV-TK, GCV mediated apoptosis occurs mainly by activating the mitochondrial damage pathway independent of p53 status (59).

1.6.3.2 HSVTK/GCV bystander effect

As explained previously, the HSV-TK system has been utilized in several clinical trials in recent years. Despite promising results *in vitro* and *in vivo*, the anti-tumour effect in clinical trials remains poor. One of the therapeutic limits remains the low percentage of tumour cells that express the suicide gene after its introduction. An *in vivo* transfection efficiency of less than 10% is common and therefore attempts are being made to increase this percentage. However, very high percentages of transfected cells are not mandatory for complete eradication of a tumour *in vivo*. Transfected tumour cells appear to be capable of inducing the death of neighboring untransfected cells; this is called the "bystander effect". In order to optimize gene therapy, one should also increase the so-called "bystander effect". This approach is based on the observation that not only the HSV-tk positive cells (effector cells), but also their neighboring HSV-TK negative cells (target cells) are killed after administration of GCV. It was in 1990 that the bystander effect of the HSV-TK/GCV system was described for the first time. Moolten and Wells (60) observed that cells transduced with the HSV-TK gene and treated with GCV induced sensitivity to neighboring non-transduced cells.

There are different factors that play a role in accomplishing a strong bystander effect. The primary major limitation of the bystander effect in HSV-TK/GCV system has been attributed to connexin 43-mediated gap junction intracellular communication, involving the passage of cytotoxic phosphorylated GCV derivatives between the cells. In this system the untransfected cells are killed when they receive the GCV-metabolites through gap junction from neighboring cells. Several studies has shown that the GCV-mediated cell killing was enhanced when more cells expressed the HSV-TK gene (61) or when more copies of HSV-TK is transfected (62), however it was shown that the increased gap junction communication is needed to enhance the bystander effect and the therapeutic efficacy (63).

1.7 Two suicide genes for cancer treatment

An established principle of antineoplastic chemotherapy is that the multidrug therapies are generally superior to single agent therapy. The first combined therapy was based on HSV/GCV and the cytosine deaminase/5-fluorouracil (CD/5-FC) systems. A cooperative effect of both suicide genes was described in 9L gliocarcoma cells (64). Another approach to combine both systems used the CD-TK fusion gene under the control of a human inducible heat shock protein 70 promoter. A significant reduction in the survival of prostate carcinoma cells was observed(65). The combination of these two suicide genes has also been shown to sensitize the tumor cells to radiation (66).

A few studies have referred to the use of CD and uracil phosphoribosyle transferase (UPRT) for cancer treatment. Two of them have demonstrated that an adenovirus expressing CD in 9L glioblastoma model extents survival rate of the animals (67). Another study combining two adenoviruses, one expressing CD and another UPRT for treating 9L rat glioblastoma, has shown an increased anti-tumor activity and an improved survival in rats (68). A similar study performed in a prostate cancer model has shown reduction in tumor growth (69).

1.8 Replicating oncolytic adenoviruses

Oncolytic virotherapy is an approach for the treatment of cancer, in which the replicating viruse itself or the genetically engeneered replicative viruses (in case of adenovirus) specifically target and destroy tumor cells via their cytolytic replication cycle. As this approach relies on viral replication, the virus can self-amplify and spread in the tumor from an initial infection of only a few cells. The success of this approach is based on the ability to deliver the replication-competent viral genome to target cells (91).

1.8.1 Oncolytic adenoviruses and gene therapy

Many oncolytic adenoviruses have the cloning capacity for a small transgene. Addition of an auxiliary therapeutic transgene could augment killing of the infected tumor cells and the nearby tumor cells via bystander effect. One group of therapeutic genes that have received much attention in virotherapy is the suicide genes (prodrug-activating genes). In the case of HSV-TK gene the nontoxic prodrug (GCV) could be converted to its toxic form as previously discussed. The toxic metabolite inhibits the viral and cellular DNA synthesis and can spread to untransduced tumor cells via gap junction, thereby enhancing the killing effect beyond the infected cell (bystander effect). Targeting gene- virotherapy combines the advantages of gene therapy and viral therapy.

Virotherapy is being re-evaluated based on the findings by Bischoff et al (70) demonstrating that the E1B-55KDa-deleted adenovirus (ONYX-015), replicates in and lyses p53-dysfunctional cells. This virus that does not express a therapeutic gene has a potential to replicate preferentially in tumour cells. It has been used in clinical trials in patients with advanced carcinoma of head and neck (71). Scientists have combined the advantages of the prodrug/suicide gene approach with the E1B deficient adenoviral vector to develop E1B 55 KDa-deficient adenoviral vectors expressing HSV-TK (72) or carrying a cytosine deaminase-HSV-TK fusion gene (73). In Ad-CD/Tkrep that uses a double-suicide transgene approach, the cytosine deaminase converts 5-fluorocytosine into 5-fluorouracil, which has antitumor activity. It has been shown that the antitumor effect of this virus is enhanced when used in combination with GCV (intravenously) and 5-fluorocytosine (orally) in vivo and in vitro compared with the replicative virus alone or in combination with a single prodrug (73). Furthermore, the addition of radiation therapy to GCV, 5-fluorocytosine, and virotherapy led to greater tumor growth delay and a 80% complete response that was superior to other treatment strategy (74). For future vector developments it will be essential to achieve maximum vector distribution and transgene expression within tumors and to trigger the immune response against the cancer cells.

1.9 Goals of this work

A recent publication has shown that the coexpression of thymidine kinase (TK) and guanylate kinase (GK) enhances vascular smooth muscle cell killing and permits administration of GCV at lower doses when compared to the expression of thymidine kinase alone (75).

Based on that publication, we evaluated the cytotoxic effect of the recombinant adenoviruses coexpressing two suicide genes: TK and GK versus one expressing only the suicide gene TK. These were tested in two different cancer cell lines including the human glioblastoma (U87) and the ovarian cell lines (TOV 21G). We also tested these viruses in non-cancerous cells including normal human fibroblast cells (MRC-5) and human umbilical vein endothelial cells (Huvec). The objectives are:

1. Using the coexpression of TK with the GK gene to determine whether the second phosphorylation of GCV is the rate-limiting step in tumour cells killing.
2. To determine whether the production of a replication-competent AdV carrying the above mentioned suicide genes would improve its cytotoxic effect on cancer cells.
3. In the event that replication benefits the activity of the virus, we looked for an efficient strategy to insert the transgene in the vicinity of the E4 region (between RITR and E4 promoter) of the adenovirus, in order to have a replication competent adenovirus for cancer therapy purposes. The objective was to determine the best configuration for the insertion of the transgene in the region between E4 and RITR. This, in order to have the best transactivation of the transgene under the control of a cumate inducible promoter, and the best viral growth (titer).

Chapter 2 :
Material and methods

2.1 Construction of adenoviral transfer vectors expressing TK or co-expressing TK and GK

All the enzymes used in this study were obtained from New England Biolabs, Beverly, Mass., USA.

The GFP (Green Fluorsecent Protein) used in the construction of the transfer vectors is the GFPq of Q-biogen (Q-biogen, Montreal, Canada).

The transfer vector called adenovator that was used in this study consists of two homology regions with the left and right arm of the type 5 adenovirus (Ad5), an origin of replication (ori), a kanamycin resistant gene. The desired genes were cloned in this backbone in the presence or absence of IRES-GFP. IRES is an internal ribosomal entry site that can directly recruit ribosomal 40S subunits for initiation of translation (76).

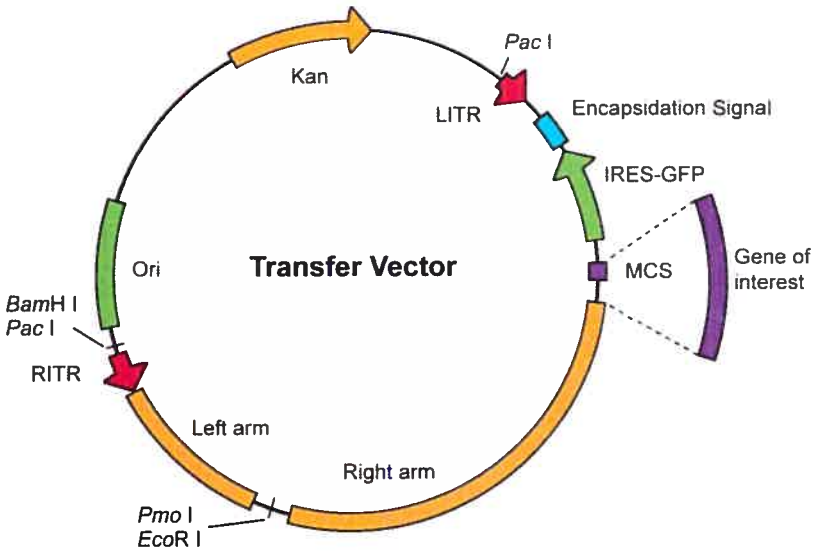


Figure 7: General map of the adenovator vector.

2.1.1 AdenoVator-CMV5(CuO)TK-IRES-GFP

The transfer vector pAdenoVator-CMV5(CuO)TK-IRES-GFP was constructed by ligating the TK fragment in *Bgl*II- *Not*I cloning sites of the pAdenoVator–CMV5(CuO)-IRES-GFP (Q-biogen, Montreal, Canada) transfer vector. The TK fragment was generated by PCR from pGT60codAupp (InvivoGen, San Diego), adding the BglII restriction site to the forward primer and the NotI restriction site to the reverse primer. The PCR amplification was performed with high fidelity Vent enzyme and its buffer in the presence of DMSO (8% of the whole PCR volume).

The PCR reaction was done using the following program:

1- Step 1	96° C/ 2 min
2- Step 2 : Cycle 1- Cycle 4	96° C/ 45 sec
	48° C/ 45 sec
	72° C/ 1: 30 min
3- Step 3 : Cycle 5- Cycle 25	96° C/ 45 sec
	65° C/ 5 sec
	72° C/ 1: 30 min
4- Step 4	72° C/ 10 min
5- Step 5	4° C (for conservation)

Then the TK fragment was isolated by cutting the corresponding 1.3 Kb band from a 1% agarose gel and purified using the " QIAEX II Agarose Gel Extraction Kit" (Qiagen Inc). Basically, the QIAEX II principle involves the extraction and purification of DNA fragments based on the solubilization of agarose with chaotropic salt and quantitative adsorption of nucleic acids to the QIAEX II silica-gel particles. Elution of DNA was performed in a suitable volume of buffer (eg., TE, pH 8.0, or 10 mm Tris.HCl, pH 8.5). The purified PCR TK was cut with the restriction enzymes *Bgl*II and *Not*I. The transfer vector pAdenoVator-CMV5(CuO)-IRES-GFP was cut in the multiple cloning sites using the same restriction enzymes. Then the cutted ends were dephosphorylated with the alkaline phosphatase enzyme and purified using "QIA quick mini columns" (Qiagen Inc). The purified TK fragment was ligated into the above mentioned purified transfer

vector using the T4 DNA ligase enzyme of *E.coli* and its T4 DNA ligase buffer and incubated overnight at 14° C. The ligation reaction product was then transformed into competent *E.coli* DH5α. Transformation was done by incubating the bacteria and the DNA for 30 min on ice. Then a heat shock was done at 42° C for 1 min, and 900 µl of LB medium was added and the whole reaction was transferred for an incubation of 1hour at 37° C in a shaker. The bacteria were then concentrated and spread out on LB agar plates supplemented with 50 µg/ml of kanamycine. Colonies were picked up, grown overnight and analyzed by restriction enzymes. The presence of the 1.3 Kb TK gene was confirmed by cutting with BglII and NotI enzymes and verified on 1% agarose gel. Also the results obtained from PCR with the above- mentioned TK primers confirmed the presence of the 1.3 Kb TK gene. The positive clones were then amplified and the recombinant DNA was extracted using Qiagen maxi- prep kit (Qiagen Inc). Basically, the QIAGEN plasmid maxi protocol is designed for preparation of up to 500 ng of high or low- copy plasmid using the QIAGEN plasmid maxi kit. In order to determine the yield of the extracted recombinant plasmid, DNA concentration was estimated by both UV spectrophotometry and quantitative analysis on a 1% agarose gel.

2.1.2 AdenoVator-CMV5(CuO)TK-cite-GK

The TK-cite-GK fragment was isolated from the pBS-TK-cite-GK vector (Kindly provided by Elizabeth G. Nable, NIH, Bethesda) using the restriction enzymes *Xho*I and *Xba*I and rendered blunt ended with the *Klenow* fragment of *E.coli* DNA Polymerase I. The relation between the TK and GK was carried out by the cite fragment, which contains a copy of the encephalomyocarditis 5' noncoding region and functions as an internal ribosomal entry site for initiation of translation by eukaryotic ribosomes. The blunted 2.5 Kb TK-cite-GK insert fragment was purified from a 1% agarose gel as previousely described. The transfer vector pAdenoVator-CMV5(CuO)mcs (Q-biogen, Montreal, Canada) was cut in the multiple cloning sites using the restriction enzyme *EcoR*V and dephosphorylated and purified. The purified TK-cite-GK fragment was ligated into the above mentioned purified transfer vector overnight at room temperature

and the transformation was performed as previously described. Colonies were picked and the desired orientation of the TK-cite-GK gene was confirmed by digesting with the *Hind* III restriction enzyme and verifying on a 1% agarose gel. Also the orientation of the insert was confirmed by sequencing with the dRhodamine kit (company), using a sense or an anti-sense oligonucleotides annealing outside of the multiple cloning site of the pAdenoVator–CMV5(CuO)-mcs. The following condition were used for the sequencing reaction:

1 μg of the cloned vector.

0.8 μl DMSO

(1.6 pmol/ml) of the oligonucleotide

4 μl dRod.seq. mix (Amersham Pharmacia biotech, San Francisco, CA)

The PCR reaction was then done using the following program:

1- Step 1	96° C/ 10 sec
2- Cycle 1- Cycle 25	96° C/ 30 sec
	50° C/ 5 sec
	60° C/ 4 min
3- Step3	72° C/ 10 min
4- Step 4	4° C (for conservation).

Once the reaction completed, the DNA had to be purified on the centri- sep column (company), and sent for gel analysis (BRI sequencing facility). The sequence obtained was compared with the expected sequence. Positive clone was then amplified and the DNA was extracted as previously described.

2.2 Construction of the adenoviral transfer vectors expressing CR5GFP cassette in the E4 complete and or E4 deleted orf6^{+} backbones

The transfer vectors named pAd that are used to generate the replicative adenoviruses are designed in two platforms. In pAdE4-orf6+ext the E4 region of the adenovirus is deleted except for the orf6 region followed by the polyA site of the globulin gene but in pAdE4ext the E4 region is not deleted. Both of the transfer vectors consist of a homology region with the right arm of the type 5 adenovirus (Ad5), an origin of replication (ori), a resistance gene to ampiciline (β- lactamase), the PacI site in replacement with the E3 region of adenovirus.

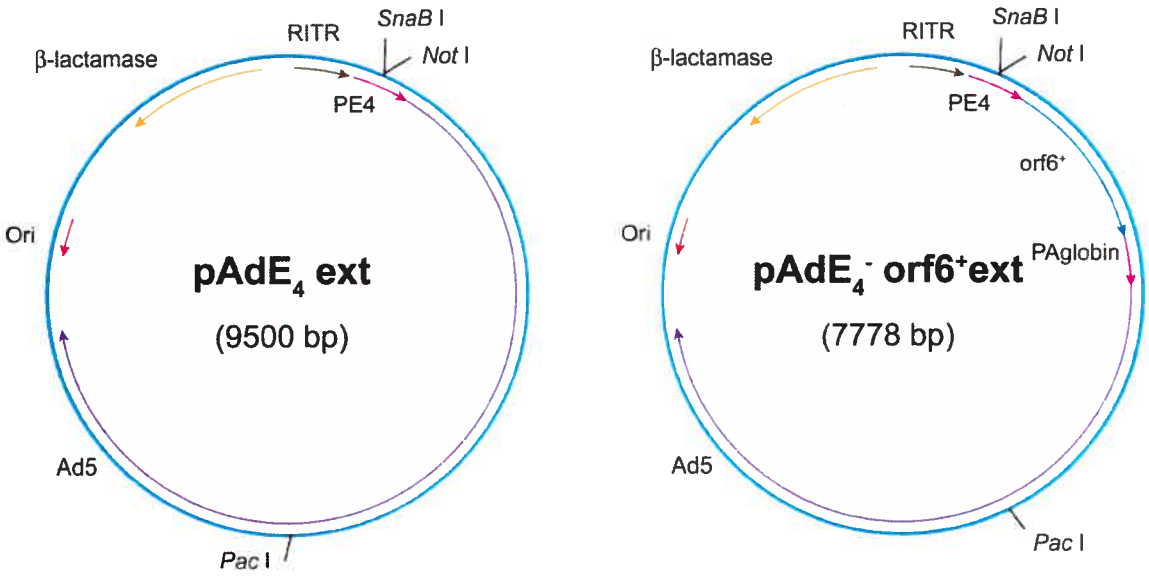


Figure 8: General map of adenoviral vectors used in this study.

2.2.1 Construction of pAdCR5GFP-R/PS$^+$ and pAdCR5GFP-L/PS$^+$

pAdCR5GFP-R/PS$^+$ and pAdCR5GFP-L/PS$^+$ were constructed by ligation of the CR5GFP cassette in *SnaB*I cloning site (165 bp from RITR) and the PS fragment in PacI cloning site of the pAdE4ext backbone transfer vector. The CR5GFP fragment was generated by PCR from pAdCR5GFP vector (33), adding the *EcoR*V restriction site at both forward and reverse primers. The PCR amplification was performed with high fidelity Vent enzyme and its buffer.

The PCR reaction was done using the following program:

1- Step 1	96° C/ 10 sec
2- Cycle 1- Cycle 4	96° C/ 45 sec
	48° C/ 45 sec
	72° C/ 1 :30 sec
3- Cycle 5- Cycle 25	96° C/ 45 sec
	60° C/ 5 sec
	72° C/ 10 sec
4- Step 4	72° C/ 10 min
5- Step 5	4° C (for concervation)

Then the PCR fragment was isolated by cutting the 1.7 Kb band from a 1% agarose gel and purified as described before. The purified PCR CR5GFP was cut with *EcoR*V. The transfer vector pAdE4ext was cut in the *SnaB*I site using the *SnaB*I restriction enzyme and dephosphorylated and purified. The purified CR5GFP was ligated into the above mentioned purified transfer and incubated overnight at room temperature and the transformation was performed as described before. The bacteria were then concentrated and spread out on LB agar plates supplemented with 100 µg/ml of ampiciline. Colonies were picked up, grown overnight and analysed by PCR to confirm the presence of CR5GFP gene. The sense and antisense orientations of the CR5GFP gene were selected by cutting with *BamH*I and *Cla*I enzymes verifying on a 1% agarose gel. The positive clones selected for both orientations of CR5GFP gene were then amplified and the recombinant DNA was extracted. The PS gene under the control of TK promoter was

then sub-cloned into the created *Pac*I site of the constructed vectors. PS fragment was isolated from the pAdCMV5DCGFP/TK-PS vector (Gagnon et al, in preparation) using the restriction enzyme *Pac*I by cutting the corresponding 700 bp band from a 1% agarose gel and purified. The pAdCR5GFP-R and pAdCR5GFP-L transfer vectors were cut at the restriction site *Pac*I using the same restricted enzyme and dephosphorylated and purified. The purified PS fragment was ligated into the above mentioned purified transfer vector and incubated overnight at 14° C. The ligation reactions product was transformed in the competent bacteria as previousely described. Also the orientation of the PS was confirmed by sequencing with the Rhodamine kit, using a sense and or an anti-sense oligonucleotides anealing outside of the *Pac*I restriction site of the pAdCR5GFP-R and pAdCR5GFP-L transfer vectors. The sequencing condition was performed as explained in 2.1.2 section and the positive clones were then amplified and the DNA was extracted.

2.2.2 Construction of pAdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$ and pAdCR5GFP-L/E4$^-$ orf6$^+$/PS$^+$

The transfer vectors pAdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$ and pAdCR5GFP-L/E4$^-$ orf6$^+$/PS$^+$ were constructed by sub-cloning of the PS fragment in *Pac*I cloning site of the pAd E4$^-$orf6$^+$ backbone transfer vector at first step and sub-cloning CR5GFP cassette in *SnaB*I cloning site of pAdE4$^-$orf6$^+$PS$^+$ transfer vector in both orientations at second step. PS fragment was cloned in the *Pac*I site of pAd E4$^-$orf6$^+$ext backbone transfer vector as described in the previous step. The desired orientation of PS was confirmed by sequencing. Positive clones were then amplified and the DNA was extracted. At the next step, the purified PCR CR5GFP was cut with restriction enzyme *EcoR*V. The transfer vector pAdE4$^-$orf6$^+$PS$^+$ was cut at the *SnaB*I site and dephosphorylated with the alkaline phosphatase and purified. The purified CR5GFP was ligated into the purified transfer vector and incubated overnight at room temperature. After transformation colonies were picked up, grown overnight and analysed by PCR to confirm the presence of CR5GFP gene. The sense and antisense orientations of the CR5GFP gene were selected by cutting with *Not*I and *Fse*I enzymes verifying on a 1% agarose gel. The positive clones selected

for both orientations of CR5GFP gene were then amplified and the recombinant DNA was extracted.

2.3 Cell culture

The human 293A cell line (77) is the stable fibroblast kidney cell line that constitutively express Ad5 E1A and E1B genes. Those cells are cultured in DMEM medium supplemented with 5% FBS serum plus 2 mM L- glutamine. 293A cells were used for propagation of the E1 deleted recombinant adenoviruses.

The 293ACymR cell line, a clone derived from 293A cell line, were stably transfected by pCymR/tk-neo by calcium- phosphate technique as will be described later and the best clones (clone 21) were selected by infecting with AdCMV5-Cuo-LacZ in presence and absence of cumate (33). This repressor-expressing cell line allows the production of viruses with reduced transgene expression. Those cells were grown in the same media as described for 293A cells.

The 293A-PS cell line, a clone derived from 293A cells, expressing the PS gene was used for generation of PS deleted AdVs. In this attempt, 293A cells were stably transfected by pHSE*PSPAV3-DC-GFPq/tk*Hygro transfer vector by calcium-phosphate technique and the cells that were resistant to hygromycin were selected as positive clones (work done in our laboratory by Castagner., 2002 unpublished). Those cells were grown in the same media as described for 293A cells.

The BMAdE1 78-42 and 220-8 cells are adherent stable cell lines drived from the A549 cells expressing the E1A and E1B genes of the Ad5(78). Those cells are grown in DMEM medium supplemented with 5% FBS serum plus 2 mM L- glutamine. The 78-42 cells were used for purification steps and since they do not have homology sequences with adenovirus, so they can not produce the revertants (RCA). They have a low rate of growth in comparison with 293A cells, so a higher number of cells should be plated for

plaque purification in order to have good identifiable plaques (1.5 times cells more than 293A cells).

The Hela-cTA cell line, a clone derived from the Hela cells that stably express the cumate transactivator cTA. Hela cells were transfected with pcDNA-3cTA (invitrogene) and selected for G418 sensitive (work done in our laboratory, unpublished) Those cells were grown in the same media as described for 293A cells.

The glioblastoma cell lines U87 were obtained from ATCC (Manasses, VA), and grown in the same media as described for 293A cells.

The TOV 21G (kindly provided by Anne-Marie Mes-Masson, Institut du cancer de Montréal) were derived from epithelial ovarian cancer (EOC) and previously characterized and shown to mimic features of the tumoral cells from which they were derived (79). These cell lines were grown in the same media as described for 293A cells.

The normal human fibroblast cell line (MRC-5) was obtained from ATCC (Manasses, VA), and grown on DMEM supplemented with 10% fetal bovine serum and 2mM L- glutamine. The MRC-5 cells were rinsed with PBS and trypsinised with 0.25% trypsin instead of 0.05 % and sub cultivated in a ratio of 1:2 to 1:5.

Human Umbilical Vein Endothelial Cells (HUVEC) were kindly provided by Maria Moreno (NRC, Ottawa) and their special medium was purchased from Cambrex Bio Science Walkersville Inc. These cell lines were grown in EGM ^{TM}-2MV-Microvascular Endothelial Cell Medium- 2 supplemented with 5% fetal bovine serum and growth factors and cytokines. For each 25 cm^2 of HUVEC cells to be subcultured:

1- The old medium was aspirated
2- The cells were rinsed with 5ml of room temperature HEPES- BSS in order to remove all the proteins of the medium that could be inactivating the trypsin.
3- 2 ml of room temperature Trypsin/ EDTA were added to the cells for 2-6 minutes until approximately 90 % of the cells were detached.

4- 4 ml of room temperature Trypsin Neutralizing Solution were added to inactivate trypsin.
5- The cells were subcultivated in a ratio of 1:2 to 1:5.

2.4 Transfection using Calcium- Phosphate ($CaPO_4$) method

7.5×10^5 of the desired cells were plated in a 60 mm petri dish one day prior to transfection. The following day the old media was replaced by 2.5 ml of the fresh media to ascertain that the cells would grow exponentially when they receive the DNA. The precipitated DNA in 0.1X sterile TE (Tris+ EDTA) was added to a tube and 30 µl of $CaCl_2$ were added drop by drop to the DNA in TE to have a final volume of 250 µl. In the second tube 250 µl of 2 X HBS (0.28 M NaCl + 0.05 M Hepes + 1.5 mM Na_2HPO_4 PH 7.5) were added and the CaCl2- DNA mix from the first tube was added drop by drop to the second tube. Then the precipitate was added drop by drop to the medium covering the cells by trying to cover as much area as possible.

2.5 Virus construction

All the recombinant viruses were constructed by the AdEasy method or the positive selection method as will be described later.

The AdTK-IRES-GFP and AdTK-cite-GK viruses were constructed using the AdEasy method (Q-biogen) as explained in 2.5.1 section. Ad(PS+)CU-IRES-E1A was constructed with the AdEasy method by first subcloning of the CU gene in the transfer vector pAdCMV5-IRES-GFP. At the second step the E1A gene, including its viral polyA, was inserted in place of GFP downstream of the IRES in order to have co-expression of CD and E1A to have a replicative-dissiminative virus (Bourbeau et al., in preparation). The AdCR5GFP-L/PS^+(E3-tk), AdCR5GFP-R/$E4^-orf6^+/PS^+$(E3-tk) and AdCR5GFP-R/PS^- were constructed and selected by positive selection method in 293A cells as explained in 2.5.2 section. The AdCR5GFP-R/PS^+ was constructed by reinserting the PS fragment in its original space as described in 2.5.3 section. The

construction of AdCMV5GFP-L/E1$^-$ has been described in details (78). AdCR5GFP-L/E1$^-$ was constructed by *in vivo* homologous recombination between overlapping sequences of linearized pAdCR5GFP transfer vector and Ad5/E1$^-$/E3$^-$ (33). Ad PTG3602 (wild type Ad5) used to generate the Ad5ΔPS and it was amplified in 293-PS cells (8). AdE3-E4$^-$orf6$^+$ was constructed via positive selection by *in vivo* homologous recombination between the pAdE4$^-$orf6$^+$ext transfer vector and Ad5ΔPS virus in 293A cells (Elahi et al., in preparation).

2.5.1 AdCMV5(CuO)TK-IRES-GFP and AdCMV5(CuO)TK-cite-GK construction via AdEasy^{TM} method

The AdEasy method (80) is a faster way for the production of recombinant AdVs as compared to the other methods. In this system the viral backbone was made into a plasmid and is used in place of the linear viral DNA. This permits to perform the homologous recombination in bacteria instead of the mammalian cells. The viral backbone (pAdEasy-1) (Q-biogen), which carries the viral sequences, in these experiments, is E1 and E3 deleted. The transfer vectors (pAdenoVator–CMV5(CuO)TK-IRES-GFP and pAdenoVator–CMV5(CuO)TK-cite-GK were linearized with the restriction enzyme PmeI, and co-transformed into the *E.coli* strain BJ5183 together with pAdEasy-1. The BJ5183 bacteria cells supplied are electrocompetent and especially prepared for high transformation and recombination efficiency. The co-transformation was performed using the electroporation method adding 40 µl of BJ5183 bacteria cells into a 2mm electroporation cuvette following by adding 1 µg of linearized transfer vector and 100 ng/µl of pAdEasy-1 vector. The transformation was done using an electroporator supplier at the setting condition of: 5 Ohms, 2.5 KV, C=0. Immediately after the transformation mix were resuspended in 1ml of LB and incubated for 1hour at 37° C in a shaker. The reaction mixture was then concentrated and spread out on LB agar plates supplemented with 50 µg/ml kanamycin. The recombinant plasmids were selected on kanamycin plates and screened by restriction enzyme analysis with *Pac*I. The ampiciline resistant pAdEasy-1 vectors will not grow on Kanamycin plates. Then, the

selected recombinant plasmid was transformed in the competent bacteria, *E.coli* DH5α, using the heat shock method as previously described to have high yield of amplification of the recombinant plasmid. The resulting recombinant adenoviral vectors (pAdCMV5(CuO)TK-IRES-GFP and pAdCMV5(CuO)TK-cite-GK) were then cleaved with *Pac*I to linearize the viral DNA and remove plasmid sequences and purified with Phenol/$ChCl_3$ and precipitated with ethanol (Figure 9). Transfection was done with the calcium- phosphate technique as will be described later into 293 CymR cells to generate viral particles. The cytopathic effect of the produced viruses (AdCMV5(CuO)TK-IRES-GFP and AdCMV5(CuO)TK-cite-GK) on the transfected 293 CymR cells were observed 2-3 weeks post transfection (see fig 9). The lysate mixture were frozen and thawed for three times to relief the viruses from the cells. To be certain about the purity of the viruses serial dilutions from 10^{-1} to 10^{-6}, were prepared from the lysate mix in a final volume of 1ml. The serial dilutions were added to the 293A cells prepared the day before at a density of $5X10^5$ cells/well in 6 well plates. The infection was done at 37° C for 5h on a rocker. Then the media was removed and cells were overlaid with 1% Sea plaque agarose in DMEM. After 6 days fresh Sea plaque agarose was overlaid on the previous layer. In 10- 12 days plaques were isolated.

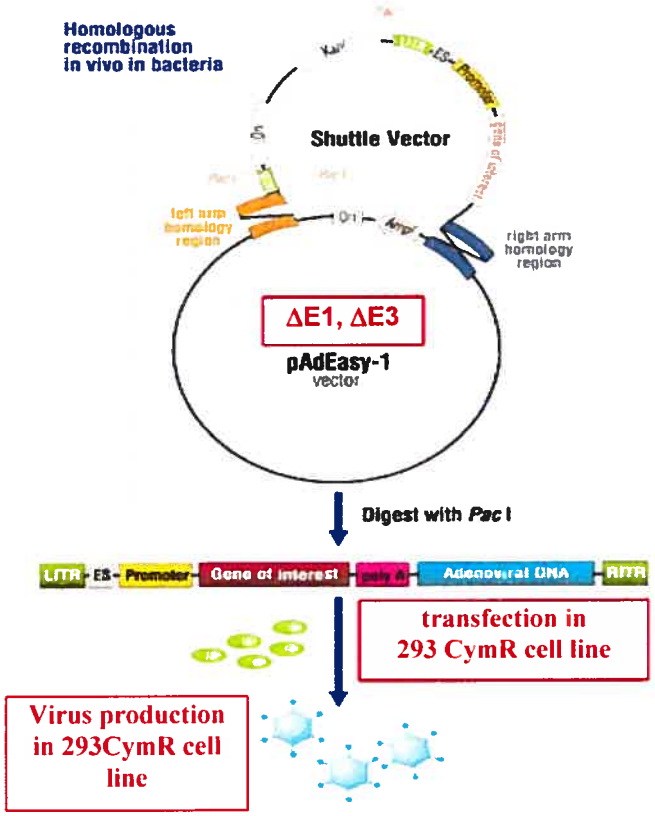


Figure 9: Production of the recombinant viruses via the Q-biogene AdEasy method.

2.5.2 Positive selection method for production of recombinant adenoviruses

The positive selection method is based on the fact that the adenoviruses deleted in the protease (PS) gene (Ad/ΔPS) are capable of only one round of replication in non-complementing cells including 293A cells (31). This feature was applied for constructing of the transfer vectors using ectopic expression of PS gene and the schema is shown in Figure 10.

In order to accomplish the positive selection, PS expressing transfer vectors were first linearized with FseI restriction enzyme overnight and purified with Phenol/$ChCl_3$ and precipitated with ethanol. Approximately 7.5 X 10^5 293A cells were plated in 60-mm dishes the day before transfection. The following day, cells were infected with the MOI of 0.1 of Ad (ΔPS) virus and incubated at 37° C on a rocker. Five hours after infection, cells were washed, fresh medium was added and cells were transfected with 5 μg of PS expressing above-mentioned transfer vectors using the $CaPO_4$ precipitation method, as will described later. The cells were washed after overnight incubation at 37° C and fresh medium was added. Three days later the transfected- infected mix was collected and frozen and thawed three times. The plaque purification assay was performed to isolate the recombinant plaques. Serial dilutions from 10^{-1} to 10^{-6}, were prepared from the transfected- infected mix in a final volume of 1ml. The serial dilutions were added to the 293A cells prepared the day before at a density of $5X10^5$ cells/well in 6 wells plate. The infection was done at 37° C for 5h on a rocker. Then the media was removed and cells were overlaid with 1% Sea plaque agarose in DMEM. After 6 days the fresh Sea plaque agarose was overlaid on the previous layer. In 10- 12 days the recombinant viruses expressing CR5GFP cassette were identified.

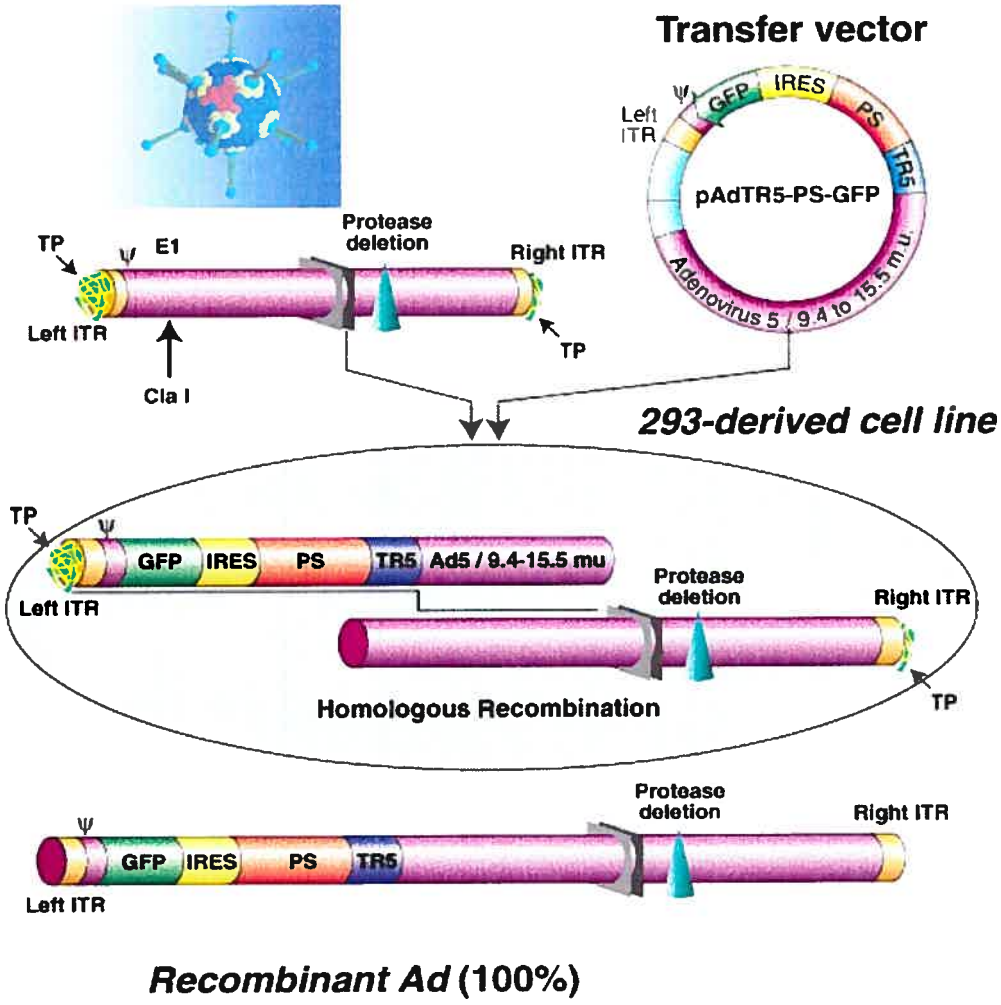


Figure 10: positive selection of recombinant AdV by ectopic expression of the PS gene (31).

2.5.3 Construction of the replicative AdCR5GFP-R/PS+

We constructed the AdCR5GFP-R/PS^{+}virus from the AdCR5GFP-R/PS^{-}. In order to return the PS fragment to it's original place, the PAdTG3602 digested by the XhoI and NdeI restriction enzymes and isolated by cutting the corresponding 5.2 Kbp band from a 0.7% agarose gel and purified with Phenol/$ChCl_3$ and precipitated with ethanol using the "QIAEX II Agarose Gel Extraction Kit". The 293A cells were then infected with the MOI of 0.1 of the AdCR5GFP-R/PS^{-} virus and transfected with the purified insert DNA via calcium- phosphate method. Afeter recombination only the

AdCR5GFP-R/PS$^+$ virus could be positively selected and develop plaques on 293A cells.

2.6 Plaque purification assay

Further purification of the selected plaques was judged necessary for the isolation of a pure recombinant virus. Each recombinant adenovirus plaque was eluted in 1 ml DMEM, and serial dilutions, from 10^{-1} to 10^{-6} was used to transduce $5X10^5$ of the desired cells in a final volume of 1ml in 6 well plates. The infection was done at 37° C for 5h on a rocker. Then the media was removed and cells were overlaid with 1% Sea plaque agarose in DMEM. After 6 days fresh Sea plaque agarose was added. In 10 to 12 days, plaques appeared and were picked and analyzed. In our experiments 293CymR cells were used to purify the AdCMV5(CuO)TK-IRES-GFP and AdCMV5(CuO)TK-Cite-GK. However, the 293A cells were used to purify all the replicative and non-replicative CR5GFP/PS$^+$ expressing viruses and the 293-PS cells were used for purification of AdCR5GFP-R/PS$^-$ virus. For all the non-replicative recombinant viruses, plaque purification was repeated on BMAdE1 78-42 cells as a result of the presence of the RCA in the viral population. 7.5 $X10^5$ BMAdE1 78-42 cells/well in 6 well plates were transduced as described above.

2.7 Viral production from the purified plaques

The amplification of the AdTK-IRES-GFPq and AdTK-Cite-GK viruses was done on 293CymR cells. Whereas the amplification of AdCR5GFP-R/PS$^+$, AdCR5GFP-L/PS$^+$(E3-tk), AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk) and AdCMV5GFP-L was performed on 293A cells and the AdCR5GFP-R/PS$^-$ on 293-PS cells. Five hundred microliters of the elutes was used to infect 5 X 10^5 cells in a 60-mm plate in 1.5ml and on a rocker for 5h at 37° C. Then media was added to have a final volume of 3ml and kept at 37° C. Once all the cells were detached, they were frozen and thawed three times, so the viruses can be released from the cells. One milliliter of this cell lysate was added to 2.5 X 10^6

cells in a 100-mm plate with 3 ml of the media on a rocker for 5h at 37° C, and then media was added to a final volume of 10 ml. The cells were gathered as previously described and finally amplified in a 150-mm plate containing 5 to 7 X 10^6 cells infected with 3 ml of the cell lysate and 5 ml of media on a rocker for 5h at 37° C and media was added to have a final volume of 15 ml. The detached cells were frozen and thawed for 3 times centrifuged at 3000 rpm for 5min and concentrated in 10 ml media and were used as a final stock for titration.

2.8 Titration of the adenovirus vectors

A virus titer is an expression of the number of infectious units per unit volume and it can be determined by different methods. The plaque assay method is the most popular method and is considered the standard but more time consuming method for viral titration. The Tissue Culture Infectious $Dose_{50\%}$ ($TCID_{50}$) method is faster, easier and less expensive but less accurate than the plaque assay method.

The titration of AdCMV5(CuO)TK-IRES-GFP and AdCMV5(CuO)TK-cite-GK viruses was performed by using the plaque assay method. This method is based on the infection of the desired cells with serial dilutions ranging from 10^{-4} to 10^{-10} of the final stock of the virus (the final amplification step). 293 CymR cells were plated at a density of 5 X 10^5 cells per 60 mm dish the day before infection. The next day, cells were infected with 500 µl of the prepared dilutions for 16 h to have maximal adsorption with agitation at 37° C. From the 10^{-7} to 10^{-9} dilutions the cells also were infected with 300 µl and 200 µl in order to have better isolated plaques and a more precise estimation of viral titer. The day after infection the medium was removed from the cells and replaced by 5 ml of 1% sea plaque agarose in DMEM. After one week the cells were overlaid with extra 3 ml of 1% sea plaque agarose in DMEM. After two weeks post-infection dishes that have less than 100 plaques were chosen and counted with unaided eye and titers were obtained using the following formula:

$$\frac{\text{Average number of the plaques}}{\text{Volume of infection X dilution}} = \text{PFU / ml}$$

PFU is the number of plaque- forming units per milliliter of original suspension.

The titration of the recombinant replicative and non-replicative viruses expressing CR5GFP cassette from a region between RITR and E4 and control replicative and first generation viruses used in this study were performed using the $TCID_{50}$ method. In this method the desired cells (293A) cells were plated at a density of 1.2×10^4 cells in 96 wells plates. The day after media was removed and infected with 50 µl of the serial dilutions of the desired virus ranging from 10^{-1} to 10^{-10} of the final stock of the virus (the final amplification step). The 8 wells of each row were infected with the same dilution stock and the row 11 and 12 were uninfected for the negative control. The infection was done at 37° C for 5h on a rocker and then fresh media were added to each well. The plates were left at 37° C for 10 days. After 10 days the number of wells including the green cells were counted in each row using the fluorescent microscope.

In order to calculate the titer the ratio of positive wells per row was calculated using the KARBER method (Manual Adeno-Quest™ version 01DE98):

For 100 µl of dilution, the titre is $T = 10^{1 + d(S - 0.5)}$

1 $d = \text{Log}^{10}$ of the dilution (= 1 for a ten-fold dilution)

2 S = the sum of the ratios

For 1ml of dilution, the titer is $T = 10^{[1 + d(S - 0.5)] + 1}$ $TCID_{50}$ / ml

It has been shown in several studies that PFU detection is less sensitive than $TCID_{50}$ assay (92; 93). The difference is due to the longer period of direct contact between the cells and the virus medium in $TCID_{50}$ assay. However, in plaque assay method the virus medium is removed after about 16h and is replaced by agarose.

2.9 Extraction of adenoviral genome (by HIRT extraction method)

The HIRT extraction is a method of extracting the small extra chromosomal DNA in the cell such as viral DNA or Plasmid (94).

293A or 293 CymR cells were plated at a density of 2.5 X 10^5 cells per 12 wells dish the day before infection. The next day cells were infected with 300 µl of eluted plaques or 100 µl of the amplified stock and kept at 37° C. Once all cells were infected and detached, they were collected to an eppendorf and washed once with PBS and centrifuged in a micro centrifuge at 3000 rpm for 3 minutes. The pellet was then re-suspended in 250 µl of TE and the lysis buffer were added to the mixture and incubated at 37° C for 2 hours :

SDS	0.6 % final
EDTA	10 mM final
Proteinase K	200 µgr/ml final

At the next step the suitable volume of NaCl 5M were added to have a final concentration of 1M in order to precipitate the cellular genomic DNA. The mixture were incubated on ice for at least 2 hours and centrifuged in a micro centrifuge at 13000 rpm for 30 minutes. The supernatant containing the viral genomic DNA were isolated carefully and purified with Phenol, Phenol/$ChCl_3$ and $ChCl_3$ at the equal volumes and precipitated with 100 % and 70 % ethanol. The precipitated viral DNA was then re-suspended in sterile water or TE.

2.10 Western blotting

This method was used to verify the expression of the proteins from the constructed recombinant adenoviruses.

Two days after infecting cells with the suitable recombinant adenoviruses, cells were collected and washed twice with PBS and centrifuged in a micro centrifuge at 5000 rpm for 3 minutes. The pellet was then re- suspended in 250 µl of Laemmli buffer (10 mM

Tris- HCl, pH 7.4, + 150 mM NaCl, 1% triton X-100, 0.25% SDS, 1 mM EDTA) and sonicated to shear the chromosomal DNA and to overcome the viscosity. The protein samples were then dosed using the DC Protein Assay micro plate protocol from Bio-Rad laboratories (Hercules, CA). The absorbency was read using a micro plate reader from Dynatech MR 5000 at a wavelength of 590 nm. The samples were diluted with Laemmli reducing 2x buffer (120 mM Tris- HCl, PH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol, 0.01% Bromophenol Blue) to have 0.5- 1 μg total protein. Diluted samples were heated at 95° C for 3- 4 minutes and loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) 10- 14% gel acrylamide Tris- glycine gels (NOVEX) that were run using 125 volt for 3 hours. Following electrophoresis, gels were transferred onto nitrocellulose Trans- Blot membrane (Bio- Rad Laboratories, Richmond, CA) at a 275 mA for one hour. The transfer buffer consisted of 25 mM Tris-HCl, 192 mM glycine and 25%, of the final volume, ethanol. The non- specific sites were then blocked by incubating the membrane in a solution of 4% nonfat dry milk (Nestle, Don Mills, Ontario) in TBS (100 mM Tris- HCl, pH 7.5 + 9.9% NaCl) with 0.1% Tween 20 overnight at 4° C on a rocker for TK protein and 2 hours at room temperature for GK protein. The membrane was then incubated with the primary antibody using the recommended dilution. TK antibody (kindly provided by William C. Summers, Yale university) was used in a dilution of 1/1000 and incubated for 2- 3 hours in a solution of 3 ml TBS- Tween 20 at room temperature using a rocker and GK antibody (purchased from Nordic immunological labratories) was used in a dilution 1/50 and incubated overnight in a blocking solution (9ml TBS- Tween 20 + 1 ml 5% milk). The membrane was then washed (3x 10min) with TBS- Tween 20. The membrane was then incubated with second antibodies; HRP-coupled anti rabbit serum diluted 4/10000 for TK and GK proteins, for one hour at room temperature using a rocker. The membrane was then washed, as mentioned earlier, and the signals were detected using the ECL chimioluminescent detection kit (Amresham Pharmacia biotech, San Francisco, CA). It is a light emitting non- radioactive method for detection of immobilized specific antigenes, directly or indirectly with horseradish peroxide (HRP) labeled antibodies.

Kodak BIOMAX MR films (Eastem Kodak Rochester, NY) were used to register the signals.

2.11 Binary dilution of the prodrug (DDD)

Cells were plated at a density of $5X10^3$ per well in 96 well plates. The following day, cells were transduced with the appropriate multiplicity of infection (MOI) in 50 µl for 4-5h, and binary dilution of GCV starting at 8.3 or 83.3 µM (depending on the cell line) were added (50 µl). Cells were incubated for 6 days and analysed by MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide](Sigma, Oakville, Canada) assays. Each condition was performed with 8 replicates and each experiment was done twice. The concentration of GCV was demonstrated in µg/ml in all the figures and was calculated using the MW of GCV (255.5 g) to convert the µM to µg/ml.

2.12 Bystander assay

Cells were plated at a density of 2 $X10^5$ in 12 well plates. The following day, they were transduced with the appropriate vector at the proper MOI. After 5h transduced cells were trypsinized and resuspended in 2 ml of media. Doubling dilution of transduced cells were prepared and added on to 96 well plates in 50 µl. The non-transduced cells (bystander cells) were trypsinised and diluted to have 2 $X10^5$ cells/ml, and added to the wells with transduced cells in 50 µl. The non-transduced dilutions were prepared with or without GCV at the appropriate concentration. The ratios of the transduced cells are as following: 50%, 33%, 20%, 11%, 6%, 3%, 1.5%, 0.8%, 0.4%, 0.2%. The cells were incubated for 6 days and analyzed by MTT assay. Each condition was performed with 8 replicates and each experiment was done twice.

2.13 MTT assay

Cells were incubated with colorometric MTT [3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide](Sigma, Oakville, Canada) at a final concentration of 1 mg/ml for 4h. The medium was removed and replaced by DMSO. Optical density was measured at 490 nm. Cell viability is proportional to the absorbance at the test wavelength (490 nm). MTT assay measures the number of living cells and is an indirect measurement of cell killing.

2.14 Guanylate kinase enzymatic assay

The guanylate kinase enzymatic assay involves the indirect measurement of both ATP and GDP formed from ATP and GMP by coupling the above reaction to the pyruvate kinase and lactate dehydrogenase reaction. The decrease in absorbancy or increasing in transmittance (transferring the light through the sample) at 340 nm associated with the NADH oxidation and is measured with a spectrophotometer using a blue filter (81).

$$ATP + GMP \leftrightarrows ADP + GDP$$

$$ADP, GDP + 2\ PEP \leftrightarrows ATP, GTP + 2\ Pyruvate$$

$$2\ Pyruvate + 2\ NADH + 2\ H^{+} \leftrightarrows 2\ lactate + 2\ NAD^{+}$$

2.5×10^6 293A and HeLa cells were plated in 100mm dishes. The Cells were infected with AdV CMV5(CuO)TK-IRES-GFP and AdVCMV5(CuO)TK-Cite-GK at MOI of 10 for 293A cells and MOI of 20 for Hela cells. The 293A cells were collected 28-29 hours, but the Hela cells were collected after 48 hours post- infection. The collected cells were washed twice with PBS and centrifuged in a micro centrifuge at 5000 rpm for 3 minutes. The pellet was then re- suspended in 500 µl of the following solution buffer (0.1 M Tris-HCl buffer + 0.1 M KCl + 0.01 M $MgCl_2$).

Final concentration

Stock Solutions (1 ml reaction volume)

1. Tris- HCl buffer, 1M, pH 7.5	0.1 M
2. KCl, 1M	0.1 M
3. $MgCl_2$, 0.1 M	0.01 M
4. Sodium Phosphoenol Pyruvate, 15 mM	1.5 mM
5. Pyruvate Kinase, 25 units/ml	2.5 units
6. Lactate dehydrogenase, 33 units/ml	3.3 units
7. NADH, 1.5 mM	0.15 mM
8. ATP, 40 mM	4 mM
9. GMP, 1 mM	0.1 mM

With the exclusion of GMP, equal volumes of the rest of the above mentioned reagents were added to form a GMP kinase reaction mixture (GKRM). 0.8 ml of GKRM was pipetted into 1ml cuvette and the transmittance were recorded at 340 nm. At the time when the transmittance (T_{340}) reading was being unchanged, the extracted sample solutions (0.1 ml) was added to the cuvette containing GKRM and the rate of change for (T_{340}) was recorded every 20 seconds for 5 minutes at room temperature. Any contaminating enzymes that may degrade ATP, PEP, or NADH result in a constant rate of decrease in $A_{340\ absorbance}$ or the increasing rate in (T_{340}), which represents a background rate. After having a steady background, 0.1 ml GMP (1mM) were added to the cuvette to initiate the guanylate kinase reaction. The difference between the increasing rate of (T_{340}) after adding GMP and the background rate represents the rate of guanylate kinase activity expressed as ΔT_{340} per minute.

2.15 Flow Cytometry analysis

Flowcytometry analysis for GFP expression was performed on a Coulter ™ XL-MCL flow cytometer (Beckman-Coulter, F) equipped with 15mW at 488nm argon ion laser as an excitation source. The green fluorescence emission was detected using a 550 nm dichronic long pass and a 525 nm band pass filter set. 10,000 cells gated on forward and side scattering were analyzed per sample. Transduced cells were transferred into a

special tube for flowcytometry and 0.2 ml of 10% paraformaldehyde were added to the samples and incubated for 30 min at room temperature. The samples were filtered and analyzed for the expression of GFP.

Chapter 3:
Results

3.1 Construction of pAdCMV5(CuO)TK-IRES-GFP and pAdCMV5(CuO)TK-cite-GK transfer vectors

Construction of the pAdCMV5(CuO)TK-IRES-GFP and pAdCMV5(CuO)TK-cite-GK transfer vectors was done using the AdEasy method as described in detail in material and methods (see 2.5.1). The construction of these recombinant AdVs was a two step process, in which the desired expression cassette is first assembled into an adenovator transfer vector, and subsequently transferred into the supercoiled adenoviral genome (pAdEasy-1) by *in vivo* homologous recombination in bacteria. The wild type adenovirus has been modified to produce the AdEasy-1 recombinant virus vector. Regions of the viral genome has been deleted (ΔE1, ΔE3) to prevent the lytic infection and also to make space for foreign DNA since a maximum of 38 Kb can be encapsidated. Schema of the transfer vectors are shown in Figure 11.

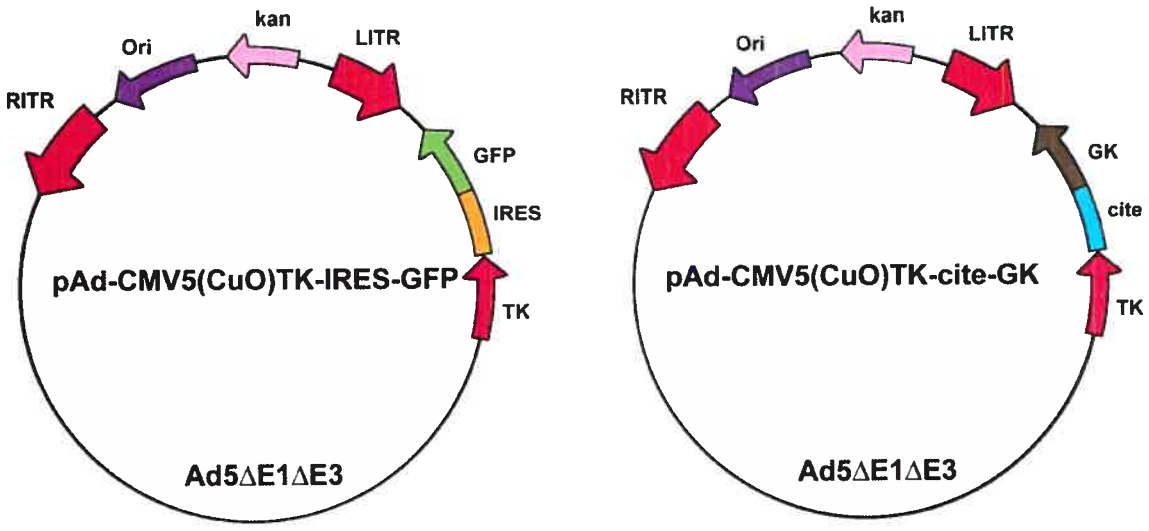


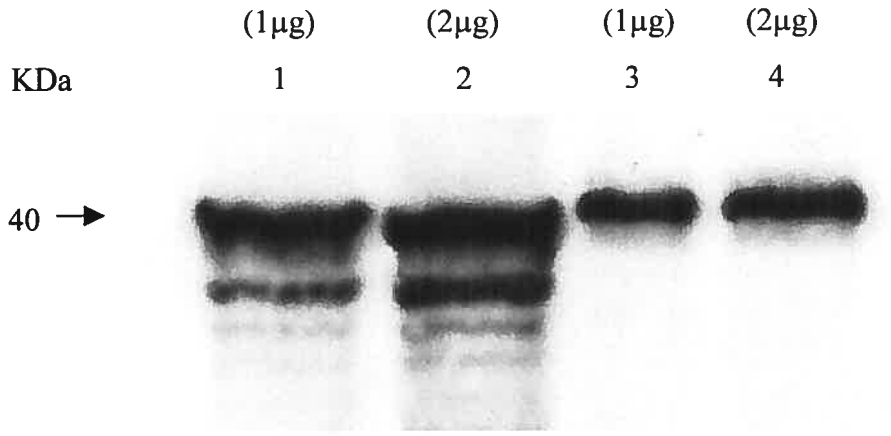
Figure 11: Schema of the transfer vectors used in this study.

3.1.1 Evaluation of cytotoxic activity of 293A cells transfected with pAdCMV5(CuO)TK-IRES-GFP and pAdCMV5(CuO)TK-cite-GK

At the first step before construction of the recombinant viruses the expression of TK protein and the cytotoxic activity of our suicide genes from pAdCMV5(CuO)TK-IRES-GFP and pAdCMV5(CuO)TK-cite-GK transfer vectors were evaluated using a bystander assay. For western blotting two different protein loadings (1 and 2 μg) were used to ascertain that the blots were not saturated and that quantification and comparison were sufficiently reliable. The results of western blot showed that the level of TK expression from both transfer vectors as almost the same with slightly higher level of expression from pAdCMV5(CuO)TK-IRES-GFP transfer vector (Figure 12). The higher TK expression from pAdCMV5(CuO)TK-IRES-GFP could be due to the difference in the number of transfected cells (transfected with pAdCMV5(CuO)TK-IRES-GFP and or pAdCMV5(CuO)TK-cite-GK transfer vectors), that could occur by transfection method. At the next step the bystander assay was performed to determine whether our genes are functional or not and to get an idea about the difference in the cytotoxic activity of TK alone as compared to the co-expression of TK and GK in presence of GCV.

The bystander activity was assessed in 293A cells by mixing the transfected cells (transfected with pAdCMV5(CuO)TK-IRES-GFP and or pAdCMV5(CuO)TK-cite-GK transfer vectors) with non-transfected cells. The results of the bystander activity using different concentrations of GCV showed that our suicide genes are perfectly active but did not demonstrate any difference in cytotoxic activity between two transfer vectors carrying TK-cite-GK and TK-IRES-GFP cassettes (Figure 13A). In comparison between the IC_{50} (the required percentage of transfected cells to kill the 50% of cells) of the above mentioned transfer vectors, we have demonstrated that almost the same amount of transduced cells were required (Figure 13B). These results showed that the co-expression of a second suicide gene (GK) with TK gene has no improvement in cytotoxic effect with the transient transfection. In order to get more solid results for better conclusion, the

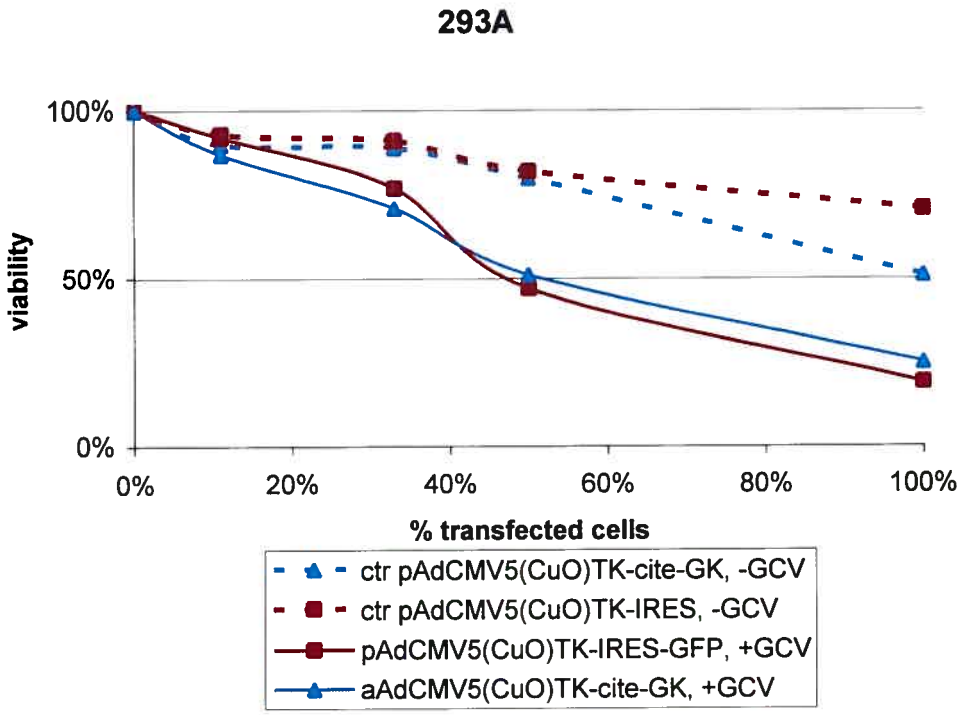
next step would be the construction of the AdTK-IRES-GFP and AdTK-cite-GK viruses from the transfer vectors and the cytotoxic activity of the viruses would be evaluated.



1, 2: 293A cells / pAdCMV5(CuO)TK-IRES-GFP
3, 4: 293A cells / pAdCMV5(CuO)TK-cite-GK
/ : Transfected by

Figure 12: Comparison of the expression of the TK protein in the 293A cells transfected with pAdCMV5(CuO)TK-IRES-GFP (lane 1 and 2) and pAdCMV5(CuO)TK-cite-GK (lane 3 and 4). The expression of the TK protein was evaluated by western blot 48h after transfection. Each sample was loaded twice on the gel 1µg and 2µg of total protein on a 12% denaturing SDS-PAGE gel. Western blot analysis was performed by standard methods using a 1/1000 dilution of the polyclonal rabbit anti-HSV-1 TK antibody. The 1/2500 dilution of the HRP-coupled anti rabbit serum were used as a second antibody.

A



B

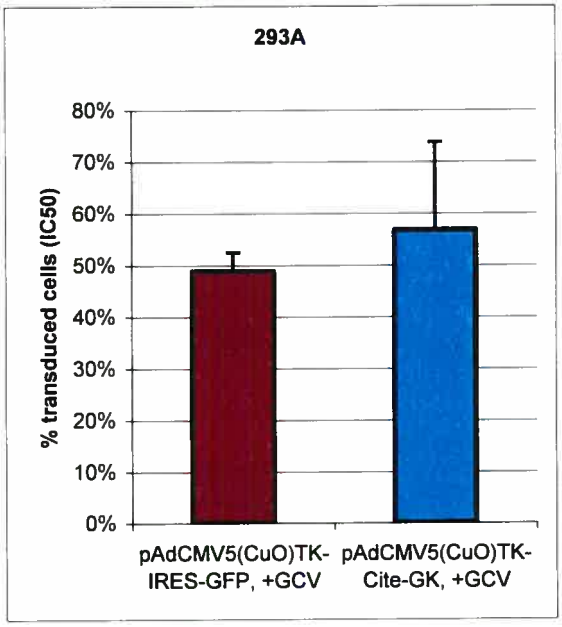


Figure 13: Comparison of the bystander activity of the transfected 293A cells with pAdCMV5(CuO)TK-IRES-GFP and pAdCMV5(CuO)TK-cite-GK transfer vectors. Cells were transfected with respective pAdCMV5(CuO)TK-IRES-GFP and

pAdCMV5(CuO)TK-cite-GK using the calcium- phosphate method. The transfected cells were mixed with the non- transfected cells at 24h post transfection at different ratios. The ratio of transfected cells was as following: 100%, 50%, 33%, 11%, and 0%. Then GCV were added at concentration of 2 μg/ml. GCV were not added to the controls using the exact ratio of transduced cells. Cells were incubated for 6 days and cell proliferation were measured using a colorimetric MTT assay. Each condition was performed with 2 replicates and the data were presented as means of two replicates plus the standard deviation. A) Curves obtained from the mean of 293A cells. B) Comparison between the ratio of the transfected cells to reach the IC_{50}.

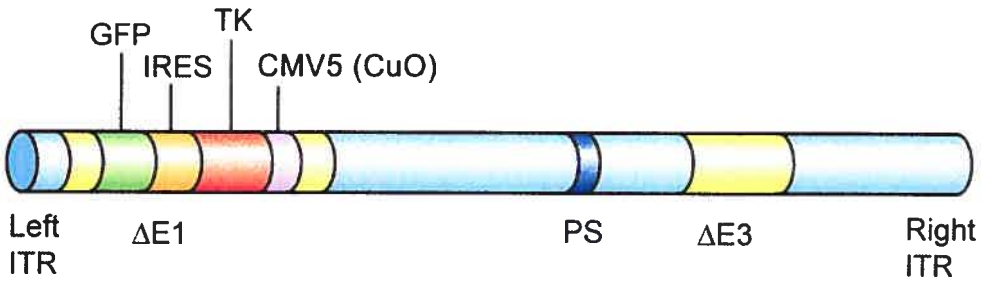
3.2 Construction of the recombinant AdVs expressing TK and co-expressing TK and GK

We have constructed two AdVs for use in cancer therapy. In order to construct these viruses, the pAdCMV5(CuO)TK-IRES-GFP and pAdCMV5(CuO)TK-cite-GK transfer vectors were linearized and 293 CymR expressing cells were transfected as described in material and methods (see 2.5.1). In our attempt to isolate the recombinant viruses in 293- CymR cell line, the co-expression of the GFP with the TK gene in the AdTK-IRES-GFP facilitated the selection of the recombinant virus plaques. The plaques were easily identified by fluorescent microscopy as green plaques on the monolayer culture that was overlaid with agarose. Then the individual plaques were analyzed by PCR of the extracted viral genome (extracted by HIRT method) for the gene of interest (TK) and the expression of the TK gene was demonstrated by western blotting (results not shown). In the case of the AdTK-cite-GK there was no GFP co-expression, so few white plaques were picked and eluted in 1 ml media. Hence, the eluted plaques were analyzed for the presence of the TK and GK genes by PCR on the extracted viral genome and demonstrating their expression by western blotting (results not shown). Finally the AdTK-IRES-GFP and AdTK-cite-GK viruses were purified, amplified and titered.

A schema of the AdVs is presented in Figure 14. Those first generation AdVs have deletions in E1 and E3 regions. The cassettes were introduced in the E1 region with an anti-parallel orientation with E1. In these viruses the expression of the transgenes from a dicistronic cassette is controlled by the CMV5(CuO) inducible promoter. In this promoter, the CuO operator sequence was placed 19 base pairs downstream of the

TATA box (between the TATA box and the initiation site). In this strategy CymR is used as a repressor that reversibly blocks the expression of the transgene from a strong promoter with the CuO operator. The CMV5(CuO) regulatory promoter allows the repression of the transcription of cytotoxic gene during AdV production in 293 cell lines expressing CymR repressor.

First generation / **AdTK-IRES-GFP**



First generation / **Ad TK-Cite-GK**

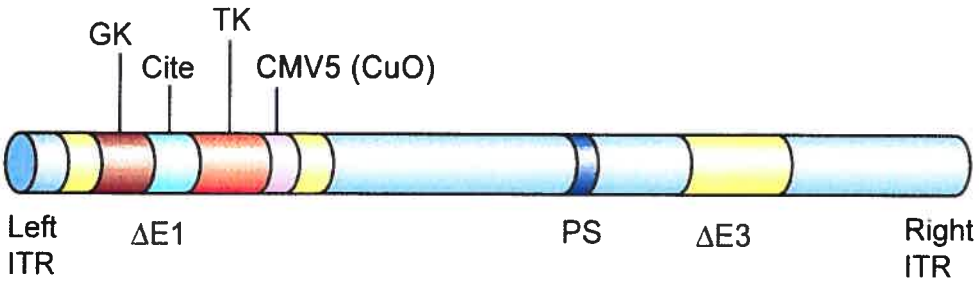


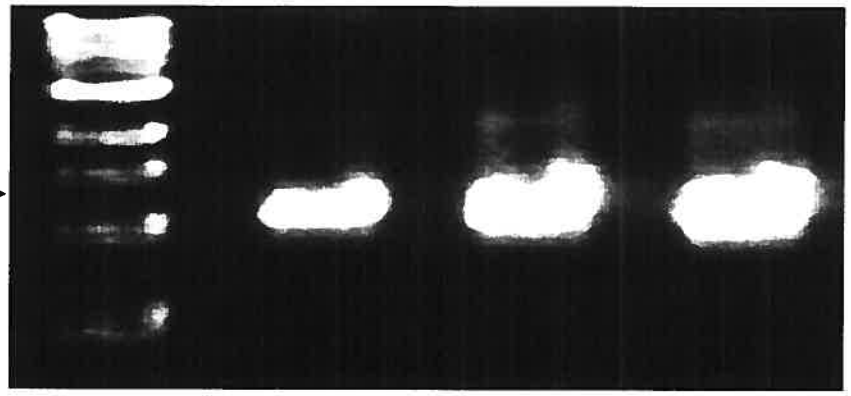
Figure 14: Schema of the first generation recombinant adenovirus vectors AdTK-IRES-GFP and AdTK-cite-GK.

3.2.1 Assessment of the recombinant AdV genotypes

In order to verify that both of the AdVs population used in this study were properly designed, PCR was carried out to test for the presence of the 1.3 Kb TK gene in AdTK-IRES-GFP and AdTK-cite-GK after being purified and amplified. The

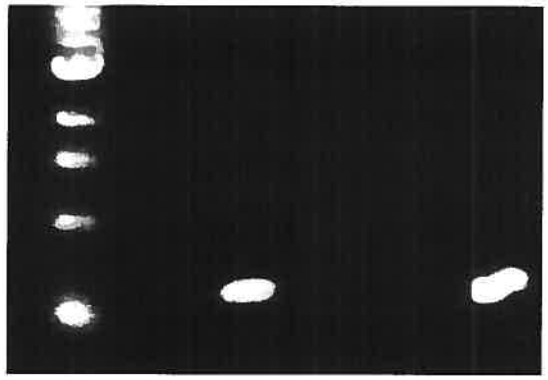
pGT60codAupp vector was used as positive control for TK gene (Figure 15A). The presence of the 593 bp GK gene in AdTK-cite-GK was also tested by PCR. The pAdCMV5(CuO)IRES-GFP vector was used as a negative control and pBS-TK-cite-GK vector was used as a positive control for GK gene (Figure 15B). The presence of $E1^+$ revertants (RCA) were tested by PCR for E1A region and the results were negative for both viruses (results not shown).

A



1 : ctr(+)pGT60codAupp
2 : AdTK-IRES-GFP
3 : AdTK-cite-GK

B

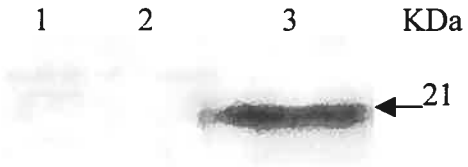


1: AdTK-cite-GK
2: AdTK-IRES-GFP
3: ctr(-) pAdCMV5(CuO)IRES-GFP
4: ctr(+)pBSTK-cite-GK

Figure 15: Adenoviruses genotypic characterization. 293A cells were transduced with AdTK-IRES-GFP and AdTK-cite-GK and the genomic viral DNA were extracted using the HIRT method. A) Genomes were tested by PCR for the presence of the TK gene. B) For the presence of GK gene.

3.2.2 Assessment of TK and GK expression from the recombinant AdVs

The TK expression from the AdTK-IRES-GFP and AdTK-cite-GK viruses was evaluated via western blotting and it was well expressed (results not shown). The expresssion of the GK gene was also evaluated by western blotting for GK protein (Firure 16). The AdTK-IRES-GFP was used as a negative control.



1: mock (non-infected)
2: AdTK-IRES-GFP
3: AdTK-cite-GK

Figure 16: Evaluation of the expression of the GK protein in the 293A cells. 293A cells were transduced with AdTK-IRES-GFP and AdTK-cite-GK at the MOI of 10. The expression of GK protein was evaluated by western blot 48h post transduction. 10 µg of each sample was loaded of total protein on a 10% denaturing SDS-PAGE gel. Western blot analysis was performed by standard methods using a 1/50 dilution of the polyclonal rabbit anti GK antibody. A 1/2000 dilution of the HRP-coupled anti rabbit serum was used as a second antibody.

3.3 Cytotoxic activity

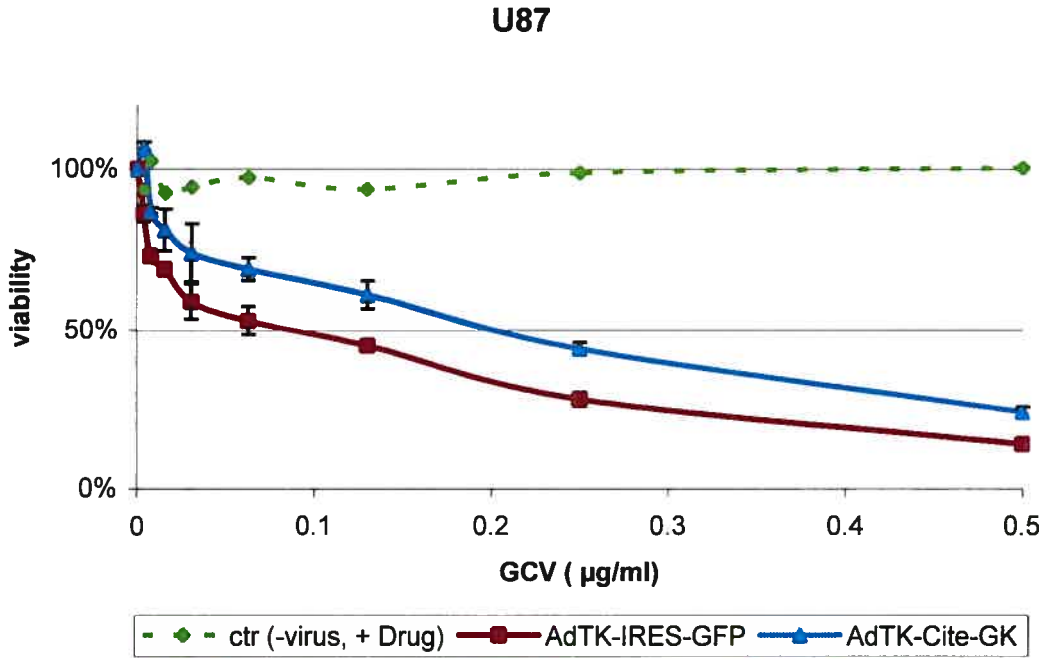
To characterize the anti-tumor activity of AdTK-IRES-GFP and AdTK-cite-GK viruses, we determined the GCV concentration required for our above mentioned AdVs to kill the glioblastoma U87 and ovarian cancer cells TOV21G. Then we determined the cytotoxic activity of the AdVs on non-cancerous cell lines including the normal fibroblast cell lines (MRC-5) and human umbilical vein endothelial cells (Huvec cells). In order to have a quantitative comparison of the vectors in all the experiments we looked for the doses of GCV that resulted in a 50% reduction in viability (IC_{50}).

The titer of the AdTK-IRES-GFP and AdTK-cite-GK viruses was determined in 293 |CymR cells under the same condition as explained in section 2.8 of material and methods and was 10^9 PFU/ml and 8 x 10^8 PFU/ml respectively.

3.3.1 Cytotoxic activity of AdTK-IRES-GFP and AdTK-cite-GK in U87 cells

In order to charactesrise the anti-tumour activity of AdTK-IRES-GFP and AdTK-cite-GK viruses in U87 cells, the cells were transduced with the respective AdVs at MOI of 20. Subsequently a set of binary dilutions of the prodrug GCV starting from 2 µg/ml to 0.004 µg/ml, were added to the cells. In U87 cells, we found that the cytotoxic activity from both viruses were similar albeit a slightly higher activity for AdTK-IRES-GFP (Figure 17A). We have determined that the IC_{50} of AdTK-IRES-GFP was 2.7-fold higher than AdTK-cite-GK (Figure 17B). The results demonstrated that co-expression of the GK gene with TK has no superior cytotoxic activity in U87 cells.

A



B

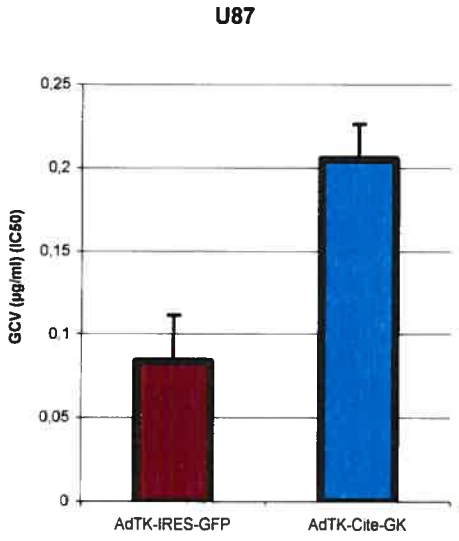


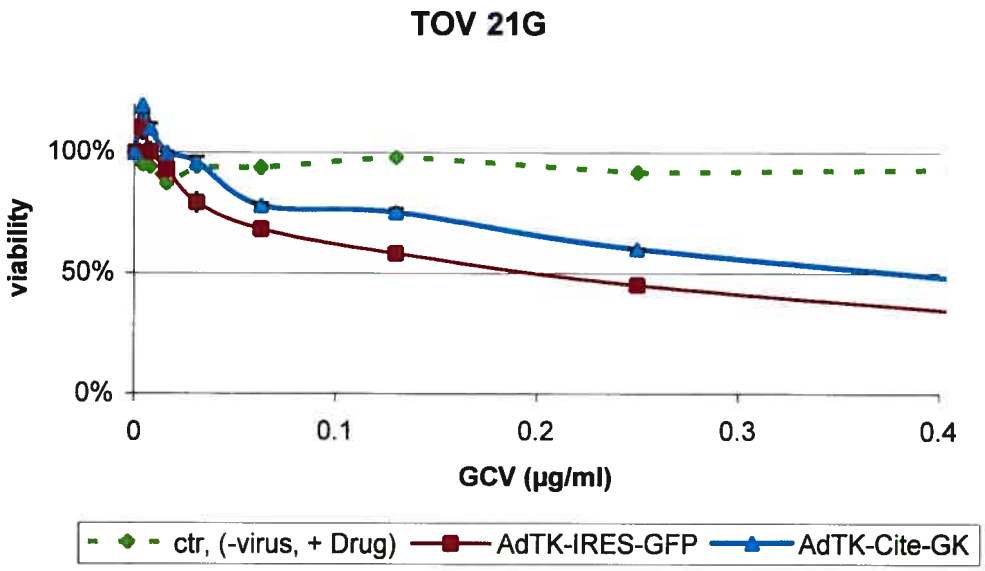
Figure 17: Comparison of antitumor activity of AdTK-IRES-GFP and AdTK-cite-GK in U87 cells. Cells were transduced with the respective AdVs at a MOI of 20. Subsequently a set of binary dilutions of the prodrug GCV starting from 2 µg/ml to 0.004 µg/ml, were added to the cells. As control, non-transduced cells were also treated with the same binary dilutions of GCV. Cells were incubated for 6 days and cell proliferation was measured using the colorimetric MTT assay. Each condition was performed with 8 replicates and each experiment was repeated twice and the data were presented as means of two experiments plus the standard deviation. A) Curves obtained with the mean of U87 cells. B) Comparision of IC_{50} at MOI of 20 for both viruses.

3.3.2 Cytotoxic activity of AdTK-IRES-GFP and AdTK-cite-GK in TOV-21G cells

In this experiment, in order to characterise the anti-tumour activity of AdTK-IRES-GFP and AdTK-cite-GK viruses in TOV 21G cells, the cells were transduced with MOI of 20. Subsequently a set of binary dilutions of the prodrug GCV at the same concentration as U87 cells starting from 2 µg/ml to 0.004 µg/ml, were added to the cells. The results showed that the cytotoxic activity using different concentration of GCV was similar with the two viruses albeit a slightly higher cytotoxic activity for AdTK-IRES-GFP (Figure 18A). The results of the IC_{50} showed a 1.5-fold higher cytotoxic activity for AdTK-IRES-GFP compared to the AdTK-cite-GK virus (Figure 18B). The results of the

cytotoxic assay in TOV21G cells showed the same results as in U87 cells. In TOV21G cells the co-expression of the GK gene with TK has no superior cytotoxic activity.

A



B

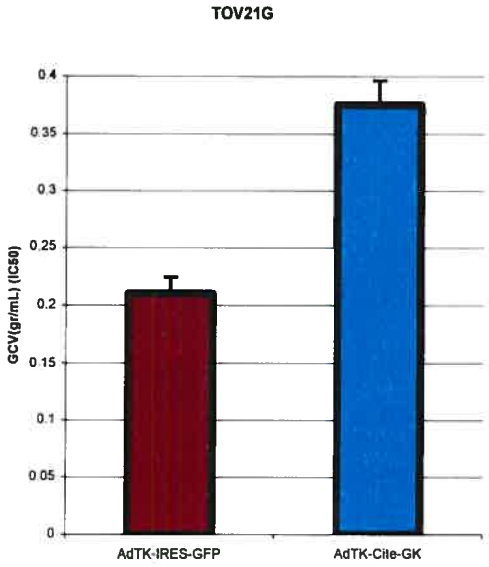


Figure 18: Comparison of antitumor activity of AdTK-IRES-GFP and AdTK-cite-GK on TOV21G cells. Cells were transduced with the respective AdV at a MOI of 20. Subsequently a set of binary dilutions of the prodrug GCV starting from 2 µg/ml to 0.004 µg/ml, were added to the cells. As control, non-transduced cells were treated with

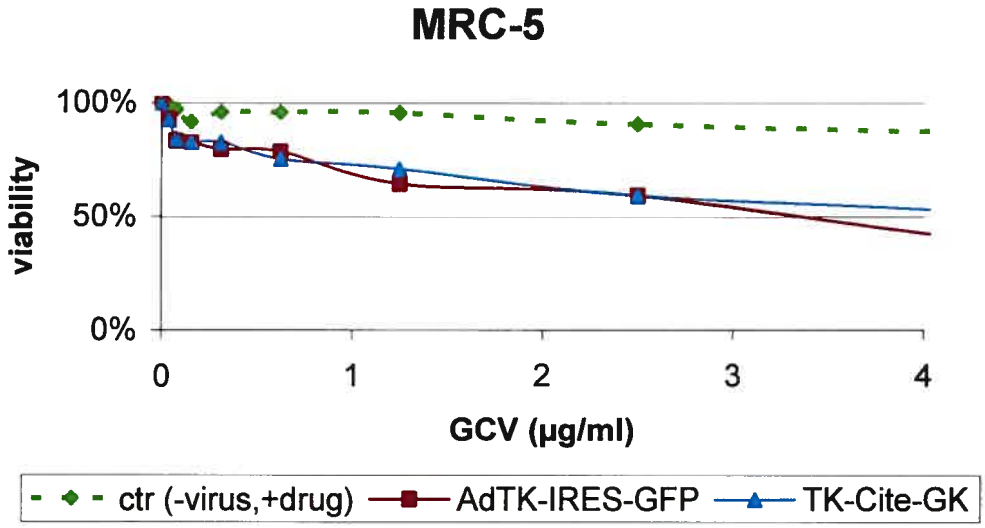
the same binary dilution of GCV. Cells were incubated for 6 days and cell viability was measured using the colorometric MTT assay. . Each condition was performed with 8 replicates and each experiment was repeated two times and the data were presented as means of two experiments plus the standard deviation. A) Viability curves obtained with the mean of TOV 21G cells. B) Comparison of IC_{50} at MOI of 20 for both viruses.

3.3.3 Cytotoxic activity of AdTK-IRES-GFP and AdTK-cite-GK on MRC-5 cells

In order to confirm the obtained results of the cytotoxic effect of AdTK-IRES-GFP and AdTK-cite-GK viruses in cancerous cell lines their cytotoxic effect was evaluated also in non-cancerous cell lines including MRC-5. The non-cancerous human fibroblast cells (MRC-5) has been derived from normal lung tissue of a 14-week-old male fetus by J. P. Jacobs in September 1966 (Nature *227:* 168-170, 1970) and is suceptible to a wide range of human viruses and supports the growth of majority of human viruses including adenoviruses. In this experiment the cells were transduced with MOI of 50. Subsequently a set of doubling dilutions of GCV starting from 20 µg/ml to 0.04 µg/ml, were added to the cells. Compared to the U87 and TOV 21G cell lines these cells were more resistant for viral infection and MOI of 50 was detected as the best MOI for evaluation of IC_{50} in MRC-5 cells. However, these cells were more resistance to GCV and the test was managed with the binary dilution of GCV starting with 10-fold higher concentration of GCV compared to U87 and TOV 21G cells.

The results demonstrated that in MRC-5 cells the cytotoxic activity of AdTK-IRES-GFP and AdTK-cite-GK viruses using different concentration of GCV did not show a remarkable difference (Figure 19 A and B). The results demonstrated that co-expression of the GK gene with TK has no superior benefit for viral cytotoxic activity in the non-cancerous MRC-5 cell line.

A



B

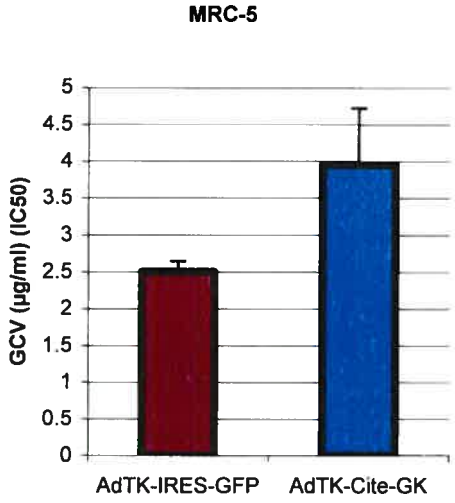


Figure 19: Comparison of the viability of the non-cancerous cells, MRC-5, transduced with AdTK-IRES-GFP and AdTK-cite-GK viruses. Cells were transduced with the respective AdVs at a MOI of 50. Subsequently a set of doubling dilutions of GCV starting from 20 µg/ml to 0.04 µg/ml, were added to the cells. As control, non-transduced cells were treated with the same doubling dilution of GCV. Cells were incubated for 6 days and viability was measured using the colorometric MTT assay. Each condition was performed with 8 replicates and each experiment was repeated twice and the data were presented as means of two experiments plus the standard deviation. A)

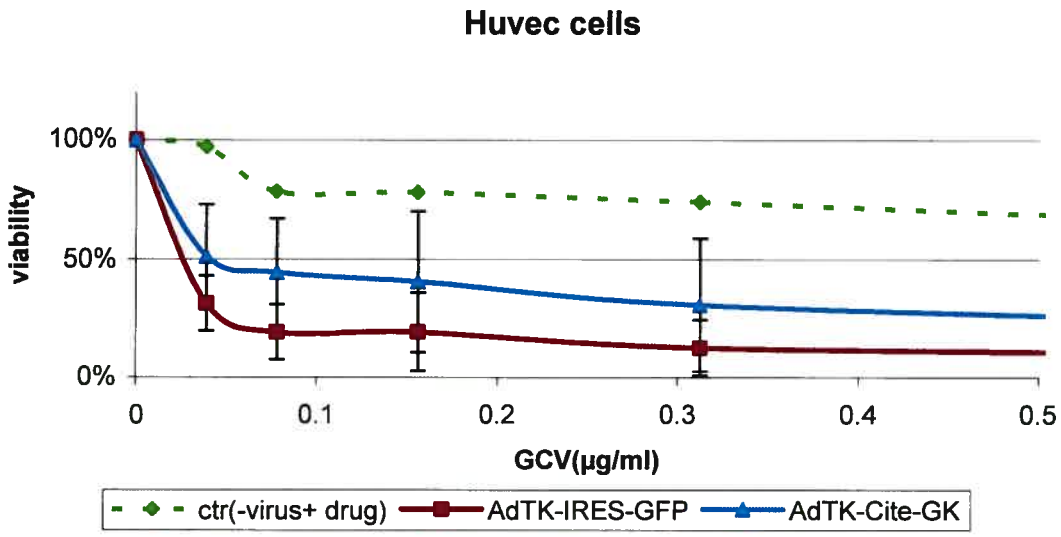
Viability curves obtained with the mean of MRC-5 cells. B) Comparison of IC_{50} at MOI of 20 for both viruses.

B

3.3.4 Cytotoxic activity of AdTK-IRES-GFP and AdTK-cite-GK in Huvec cells

The non- cancerous human umbilical vein endothelial (Huvec) cells were used to test the cytotoxic activity of our viruses. The idea of choosing this cell line was related to the recent publication that has shown that the co-expression of thymidine kinase and guanylate kinase enhances vascular smooth muscle cell killing and permits administration of GCV in lower doses as compared to the expression of thymidine kinase alone (75). In this experiment, the cells were transduced with MOI of 50. Subsequently a set of binary dilutions of GCV starting from 20 µg/ml to 0.04 µg/ml, were added to the cells. The results of cytotoxic activity of AdTK-IRES-GFP and AdTK-cite-GK viruses using different concentrations of GCV showed some better activity for AdTK-IRES-GFP virus (Figure 20A). In comparison between the IC_{50} for both viruses, we have demonstrated that 1.6 fold lower GCV concentration is required for AdTK-IRES-GFP to reach the IC_{50} compared to AdTK-cite-GK (Figure 20B). The results of the cytotoxic activity in Huvec cell lines confirmed the obtained results from three other cell lines that the co-expression of GK gene with TK does not improve the cytotoxic activity of TK gene.

A



B

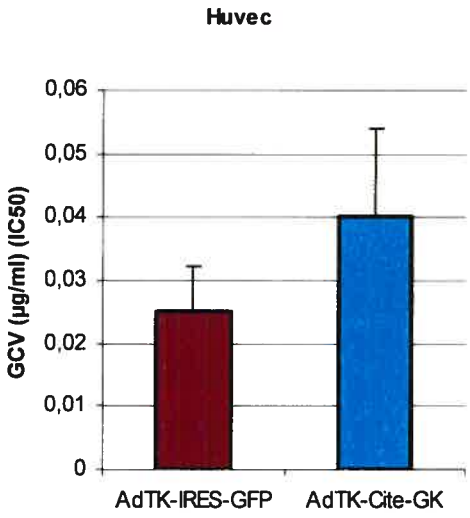


Figure 20: Comparison of the viability of the non-cancerous normal cells including Huvec cells transduced with AdTK-IRES-GFP and AdTK-cite-GK viruses. Cells were transduced with the respective AdVs at a MOI of 50. Subsequently a set of binary dilutions of GCV starting from 20 µg/ml to 0.04 µg/ml, were added to the cells. As control, non-transduced cells were treated with the same binary dilution of GCV. Cells were incubated for 6 days and viability was measured using the colorometric MTT assay. Each condition was performed with 8 replicates and each experiment was repeated twice and the data were presented as means of two experiments plus the standard deviation. A)

viability curves obtained with the mean of Huvec cells. B) comparison of IC_{50} at MOI of 50 for both viruses.

3.4 Evaluation of the TK expression from AdTK-IRES-GFP and AdTK-cite-GK in different cell lines

The previous results of cytotoxic assay in different cancerous and non-cancerous cell lines demonstrated that the co- expression of GK gene with TK had no improvement on cytotoxic activity of TK to reduce the concentration of GCV. We can hypothesize that this may be due to the lower TK expression from AdTK-cite-GK virus as compared to AdTK-IRES-GFP. In order to verify this point the expression of the TK was evaluated in MRC-5, U87 and TOV21G cells (Figure 21). The results demonstrated the same level of TK expression from both viruses in all the cell lines. The level of TK expression was also evaluated in Huvec cell line and showed the same level of TK expression (results not shown).

1: mock (non- infected MRC-5 cells)
2: MRC-5 cells // AdTK-IRES-GFP
3: MRC-5 cells // AdTK-cite-GK
4: mock (non-infected U87 cells)
5: U87 cells // AdTK-IRES-GFP
6: U87 cells //AdTK-cite-GK
7 : mock (non- infected TOV-21G cells)
8 : TOV- 21G //AdTK-IRES-GFP
9: TOV-21G //AdTK-cite-GK
//: infected with

Figure 21: Comparison of the TK expression from the AdTK-IRES-GFP and AdTK-cite-GK viruses in MRC-5 (lanes 2 and 3), U87 (lanes 5 and 6) and TOV-21G cells (lane 8 and 9). The respective cells were transduced with AdTK-IRES-GFP and AdTK-cite-GK viruses at MOI of 50 for MRC-5 cells and 20 for others. The expression of TK protein was evaluated by western blot 48h after transduction.protein samples were loaded at 2 µg of total protein on a 12% denaturing SDS-PAGE gel. Western blot analysis was performed by standard methods using a 1/1000 dilution of the

polyclonal rabbit anti-HSV-1 TK antibody. A 1/2500 dilution of the HRP-coupled anti-rabbit serum were used as a second antibody.

3.5 Assessement of the enzymatic activity of GK from AdTK-cite-GK

It was shown in the previous results that the GK protein is expressed from AdTK-cite-GK and TK is expressed at equal levels from AdTK-IRES-GFP and AdTK-cite-GK viruses in 293 A cells. We could speculate that while GK is expressed from AdTK-cite-GK, it may not be active. We thus tested its activity. The enzymatic activity of GK was evaluated in two different cell lines including 293A and HeLa cells. The 293A and HeLa cells were transduced with AdTK-cite-GK and AdTK-IRES-GFP viruses at MOI of 10 for 293A cells and MOI of 20 for HeLa cells. This GK enzymatic assay is based on the indirect measurement of both ADP and GDP formed from ATP and GMP by coupling the above reaction to the pyruvate kinase and lactase dehydrogenase reaction. The increasing transmittance at 340 nm associated with NADH oxidation was measured by a spectrophotometer using a blue filter on each sample in the absence of GMP every 20 seconds to evaluate the background rate and also in presence of GMP to evaluate the total rate. Curves were obtained according to the $\%T_{340}$ versus Time (sec) from the non-diluted 293A extracted samples (Figure 22A), diluted 293A extracted samples in a ratio of 1:3 (Figure 22B) and the non-diluted HeLa extracted samples (Figure 22C). The slope ($\Delta T/ \Delta S$) was calculated for each curve, which represent the total rate in presence of GMP and the background rate in the absence of GMP. The level of the total rate for AdTK-cite-GK in presence of GMP was the highest in all the extracts. By looking at the Figure 22B we determined that by diluting the 293A extracts the ratio of the background rate was decreased. The GK activity was calculated according to the difference between the total rate and the background rate and expressed as ΔT_{340}/min. The results of GK activity were calculated for the 293A and Hela cells transduced with AdTK-IRES-GFP and AdTK-cite-GK and summarized in Table II. The results obtained from the non-diluted 293A extracts showed 8.6X higher GK activity for AdTK-Cite-GK compared to AdTK-IRES-GFP. In 1:3 diluted 293A we demonstrated 50X higher GK activity for AdTK-cite-GK as compared to the AdTK-IRES-GFP. The

huge difference in GK activity after dilution is related to the decreasing amount of background rate after dilution. The results obtained from the non-diluted Hela extracts showed 1.7X higher GK activity for AdTK- cite-GK as compared to AdTK-IRES-GFP. The low difference in GK activity in HeLa cells is related to the high GK activity in these cells in the absence of GK expressing AdTK-cite-GK virus.

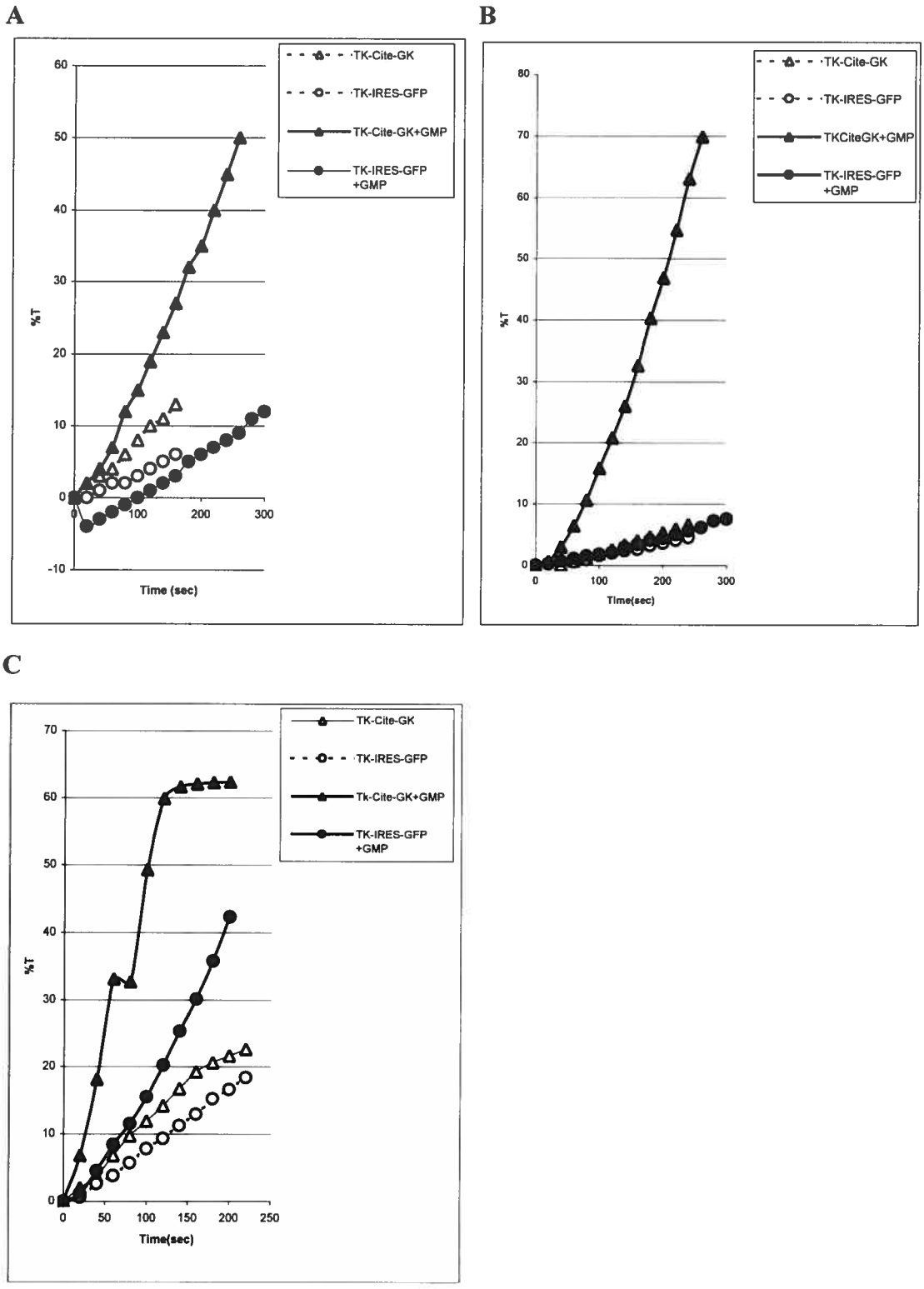


Figure 22: Evaluation of GK enzymatic activity. The 293A and HeLa cells were transduced with AdTK-cite-GK and AdTK-IRES-GFP at MOI of 10 for 293A cells and MOI of 20 for Hela cells. The transduced 293A cells were collected after 29 hours, but for the Hela cells were collected after 48 hours post infection. The activity was measured

by reading the transmittance of each sample in the absence and presence of GMP every 20 seconds for a duration of 3 minutes at 340 nm. A) Curves obtained from the non-diluted 293A extracted samples. B) Curves obtained from the diluted 293A extracted samples at a ratio of 1:3. C) Curves obtained from the non- diluted Hela cells extract.

	GK activity per min	
	TK-cite-GK	TK-IRES-GFP
non- diluted 293A extraction transfected with:	6.9	0.8
1:3 diluted 293A extraction transfected with:	15	0.3
non-diluted HeLa cells extraction transfected with:	13.86	7.51

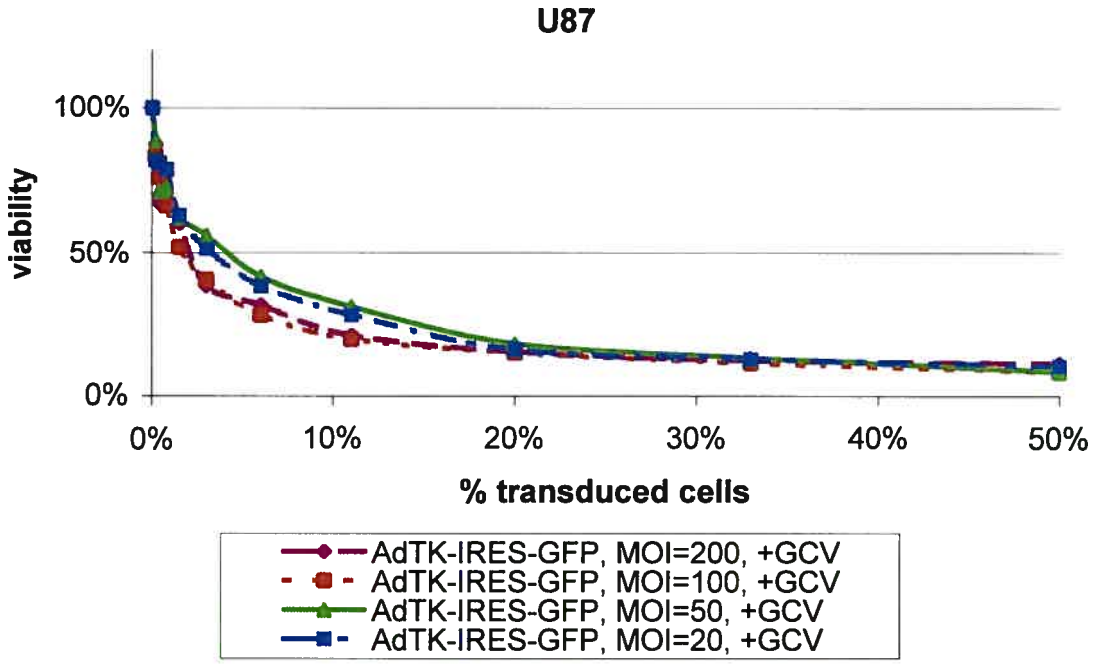
Table II: Evaluation of the GK enzymatic activity. According to the figure 22 the slope ($\Delta T/\Delta S$) was calculated for each curve. The estimated slope in the absence of GMP is represented for the background rate and in presence of GMP is represented for the total rate. The difference between the total rate and the background rate is represented by the rate of guanylate kinase activity and expressed as ΔT_{340} per minute. Therefore the guanylate kinase activity was calculated for the 293A and Hela cells infected with AdTK-IRES-GFP and AdTK-cite-GK and were compared.

3.6 Evaluation of the cytotoxic effect of increasing MOI of AdTK-IRES-GFP in U87 cells as compared to the replicative AdTK-IRES-GFP virus

In order to achieve the clinical success, having a strong bystander activity is critical. Here we tried to evaluate the potential benefit of constructing the replicative/ non-disseminative AdTK-IRES-GFP, by comparing the increasing MOI of non-replicating AdTK-IRES-GFP in U87 cells. The goal of performing this assay was to demonstrate whether the increasing MOI of the non-replicative AdTK-IRES-GFP and consequently the increasing copy number of the virus in the cells would improve the bystander effect. U87 cells were transduced with AdTK-IRES-GFP at the MOIs of 20, 50, 100 and 200. The bystander activity was assessed by mixing the transduced and non-

transduced cells at different ratios in presence of GCV. The bystander effect of the AdTK-IRES-GFP at different MOIs showed no significant difference (Figure 23A). The percentage of the transduced cells required to reach the 50% cell death was evaluated for all the four conditions (Figure 23B). The T-test was performed on the results of IC_{50} of the transduced cells at four different MOI and did not show any statistical difference. The results of western blot analysis showed that there is a correlation between the increasing in the MOI of AdTK-IRES-GFP and the increasing level of TK expression (results not shown).

A



B

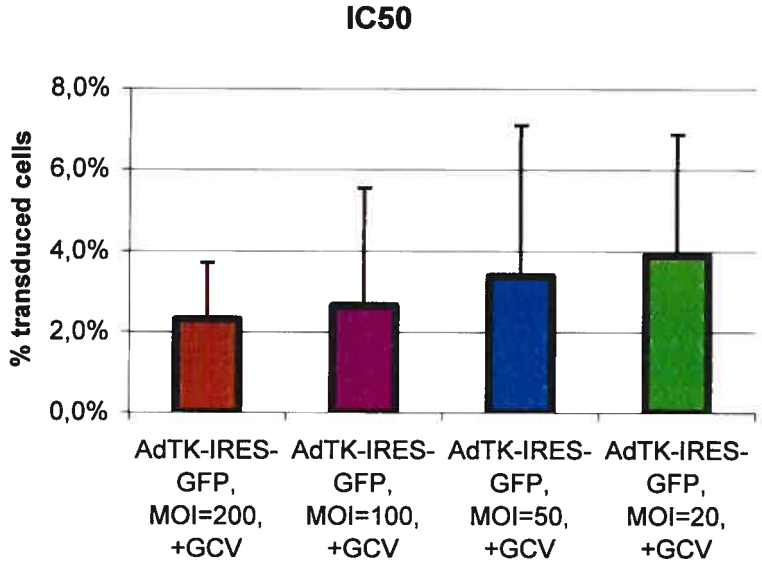
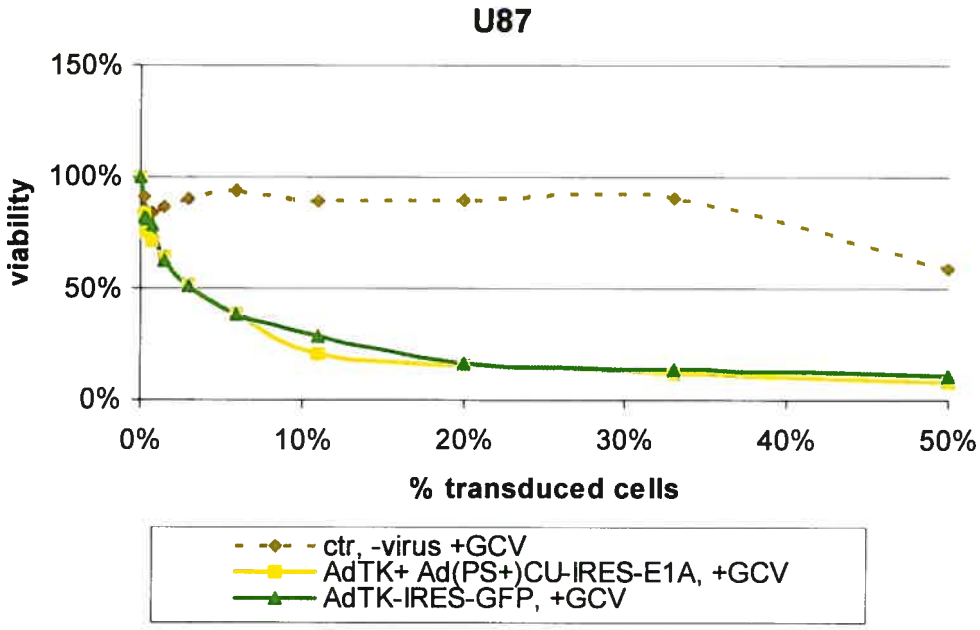


Figure 23: Comparison of bystander activity between increasing MOI of AdTK-IRES-GFP in U87 cells. U87 Cells were transduced with AdTK-IRES-GFP at the MOIs of 20, 50, 100, and 200. The transfected cells were mixed with the non- transfected cells 24h post- infection at different ratios as following: 50%, 33%, 20%, 11%, 6%, 3%, 1.5%, 0.8%, 0.4%, 0.2%. Then GCV were added at the concentration of 2 μg/ml. GCV were not added to the controls using the exact ratio of transduced cells. Cells were incubated for 6 days and cell viability were measured using a colorimetric MTT assay. Each condition was performed with 8 replicates and each experiment was performed two times and the data were presented as means of two experiments plus the standard deviation. A) Curves obtained with the mean of U87 cells. B) Evaluation of the IC50 of transduced cells.

In the other experiment we tried to confirm more directly the results from the previous experiment, the replication of the AdTK-IRES-GFP was generated by co-infection of the non-replicative AdTK-IRES-GFP with the replicative Ad(PS+)CU-IRES-GFP-E1A. U87 cells were transduced with AdTK-IRES-GFP at MOI of 20 and in the other scenario were co-infected with Ad(PS+)CU-IRES-E1A at MOI of 20. The bystander activity of both viruses was evaluated by mixing the transduced and non-transduced cells at different ratios in the presence of GCV. The bystander effect with or without replication showed no difference (Figure 24A). The T-test was performed on the IC_{50} at the desired MOI and showed no statistical difference (Figure 24B). These results

confirmed that amplifying the copy number of the AdTK-IRES-GFP via AdV replication had no significant improving effect on the anti-tumor activity in a bystander assay.

A



B

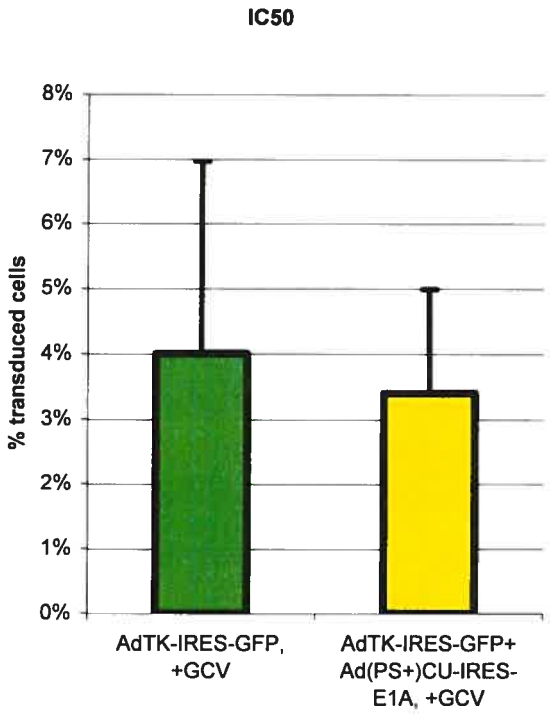


Figure 24: Comparison of bystander activity of AdTK-IRES-GFP with or without replication. U87 Cells were transduced with AdTK-IRES-GFP at MOI of 20 and in the other scenario were co- transduced with AdTK-IRES-GFP and Ad(PS+) CU-IRES-E1A at MOI of 20. The transduced cells were mixed with the non- transfected cells 24h post-infection at different ratios as following: 50%, 33%, 20%, 11%, 6%, 3%, 1.5%, 0.8%, 0.4%, 0.2%. Then GCV were added at the concentration of 2 μg/ml. GCV were not added to the controls using the exact ratio of transduced cells. Cells were incubated for 6 days and cell proliferation were measured using a colorimetric MTT assay. Each condition was performed with 8 replicates and each experiment was performed two times and the data were presented as means of two experiments plus the standard deviation. A) Curves obtained with the mean of U87 cells. B) Evaluation of the IC50 of transduced cells.

3.7 The effect of GCV on viral replication

It has been shown that the administration of GCV diminish the anti-tumor efficacy of the replication-competent HSV-TK expressing vectors in a peritoneal carcinomatosis model (82). In order to test the effect of GCV on viral growth, the 293A cells were transduced with the AdTK-IRES-GFP at MOI of 10. GCV was added at concentrations of 2 μg/ml and 10 μg/ml. We then compared the obtained viral progeny in presence or absence of GCV (Figure 25). Adding GCV at the concentration of 2 μg/ml resulted in a decrease in viral titer by about 30-fold as compared to the control. Using a higher concentration of GCV of about 10 μg/ml resulted in lower viral titer of about 60 fold compared to the control. Therefore the results showed that the increasing concentration of the GCV has a negative effect on viral replication. This can be explained by the fact that GCV acts as a virostatic agent and aborts viral multiplication. Most likely the decrease in virus progeny is due to decrease in virus rather than the decreased cell viability because the cytopathic effect of the cells starts after 3 days and by that time the production of viral progeny is completed.

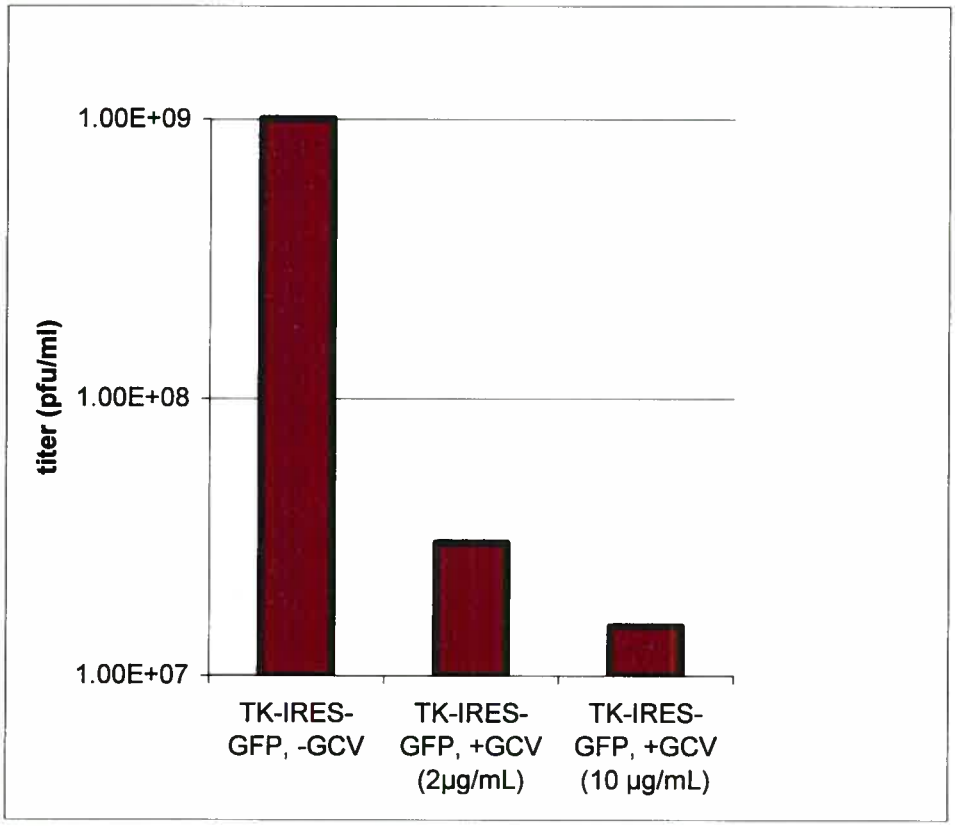


Figure 25: Evaluation of the effect of GCV on the replication of AdTK-IRES-GFP in 293A cells. 293A cells were transduced with the AdTK-IRES-GFP at MOI of 10. GCV was added at a concentration of 2 µg/ml and 10 µg/ml 3 hours post infection. Titration was performed for the cells treated with GCV and compared with the transduced cells in the absence of GCV as a control to determine the effect of the GCV on viral replication.

3.8 Construction and characterization of the replicative AdVs expressing CR5GFP cassette from a region between RITR and E4

Although replication of AdV was not beneficial to suicide gene therapy with HSV-tk, results generated in our laboratory have shown that it is very beneficial with the CU (CD::UPRT) suicide gene (Bourbeau in preparation). In order to have a replicative AdV, the E1 region of the AdV should be conserved and the transgene should be inserted in the region other than E1. Several attempts were performed in our laboratory in order to generate the replicative recombinant viruses. This part of the study was done in parallel to the previous study, which was related to the construction of the non-replicative recombinant AdVs expressing the desired suicide genes and the assessment of their cytotoxic activity. In this study we tried to find the best position and configuration to insert the transgene between the E4 region and RITR in order to allow us in future to insert our suicide genes out of the E1 region. We hypothesized that the replicative adenoviruses that produce higher level of suicide gene expression within cancerous cells could lead to more effective cytotoxic activity with lower concentrations of the prodrug.

The production of the replicative recombinant adenoviruses was a two step process. First step was the construction of the transfer vector and the second step was the construction of the recombinant virus via positive selection method. In order to construct the transfer vector, GFP under the control of CR5 (cumate inducible promoter) was inserted in a region between the RITR and the first openen reading frame (orf1) of E4 region in pAdE4ext and/ or between the RITR and orf6 of E4 in pAdE4-orf6+ transfer vector and as well the PS was inserted in the PacI site of the respective transfer vectors. In the CR5 (Cumate inducible promoter) the cumate operator (CuO) is located upstream of the minimal CMV5 promoter (TATA box). cTA is a chimeric transactivator and is formed via a fusion between the carboxylic end of CymR and the activation domain of mammalian transactivator VP16. Since the DNA binding ability of CymR is regulated by Cumate binding, it is possible to regulate its binding to DNA and hence the

transactivation of the transgene by the transactivator could be controled (see Figure 4). At the next step the recombinant AdVs were selected by positive selection method as described in matherial and methods (see 2.5.2). In this method the recombination was accomplished between the linear transfer vectors and the Ad5ΔPS as a backbone in 293A cells.

3.8.1 Possible recombinations between the tranfer vectors expressing CR5GFP cassette from a region between RITR and E4 and the Ad5ΔPS virus in 293A cells

In our platform because of the presence of the PS gene away from the transgene, there were two homology regions upstream and downstream of the PacI site of the linearized transfer vectors with Ad/ΔPS backbone virus. The pAdE4ext transfer vector has a 1.7 Kb homology region upstream and 5 Kb homology region downstream of the PacI site with Ad/ΔPS backbone virus. On the other hand, the pAdE4^{-}orf6^{+}ext transfer vector has a 1.7 Kb homology region upstream and a 2 Kb recombination region downstream of the PacI site. In theory the recombination between the homology region upstream of the ectopic PS in PacI site with the Ad/ΔPS backbone would generate a recombinant AdV carrying the ectopic PS gene including the CR5GFP expression cassette. This recombinant virus can be selected by positive selection over the Ad/ΔPS backbone on 293A cell lines (Figures 26 and 27).

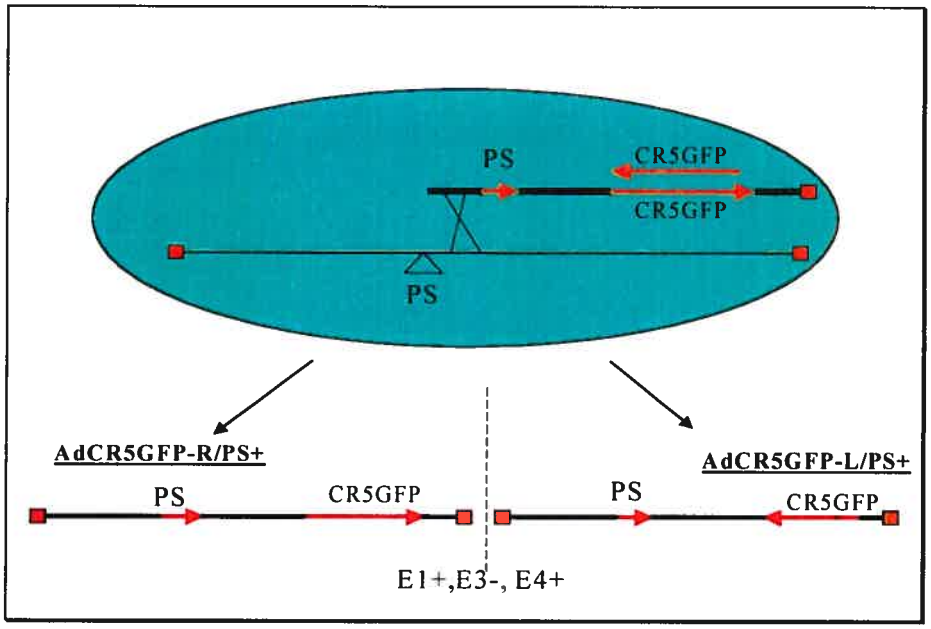


Figure 26: Schema of the expected recombination in 293A cells. Recombination between the pAdCR5GFP-L/PS^{+} (CR5GFP toward left ITR) and/ or pAdCR5GFP-R/PS^{+} (CR5GFP toward right ITR) transfer vector with the AdΔPS viral backbone and the expected resulting viruses.

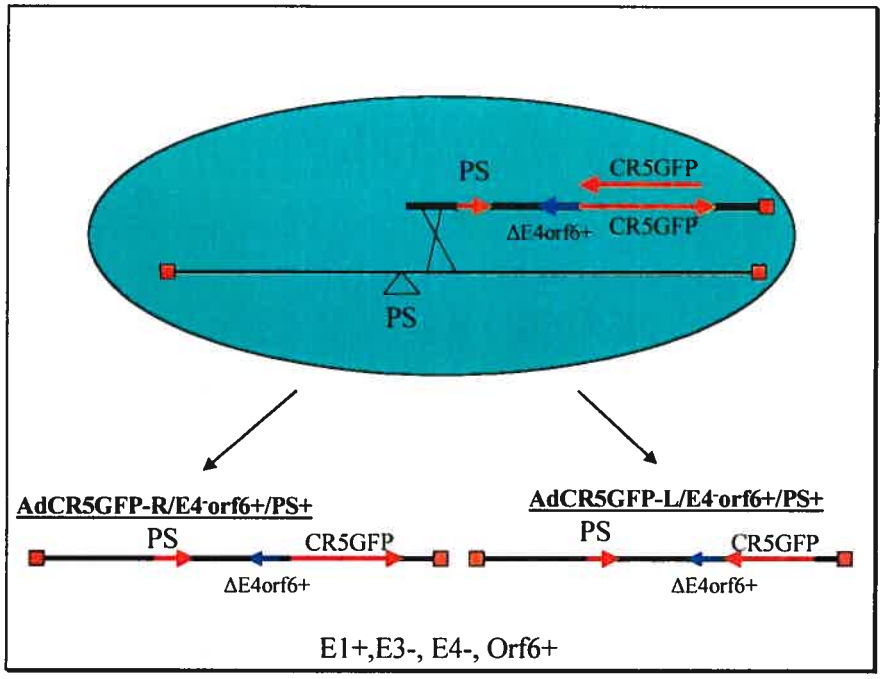


Figure 27: Schema of the expected recombination in 293A cells. Recombination between the pAdCR5GFP-L/E4^{-}orf6^{+}/PS+ (CR5GFP toward left ITR) and/ or pAd pAdCR5GFP-R/E4^{-}orf6^{+}/PS+ (CR5GFP toward right ITR) transfer vector with the AdΔPS viral backbone and the expected resulting viruses.

Furthermore, the recombination between the homology region downstream of the ectopic PS in PacI site with Ad/ΔPS backbone virus would give an AdV lacking the ectopic PS gene but carrying the transgene cassette (recombination 1 in Figures 28 and 29). This recombinant virus is dependent on the PS positive virus or the PS expressing cell line (293-PS) for its growth. However the double recombination between both sides of the ectopic PS in PacI site of the transfer vector with Ad/ΔPS backbone virus would result in having an Ad/PS+ virus lacking the transgene (recombination 1 and 2 in Figures 28 and 29).

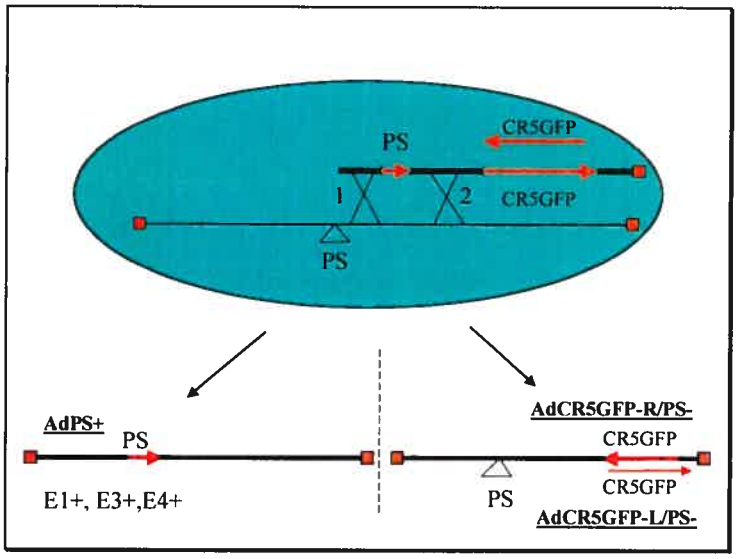


Figure 28: Schema of the possible double recombinations (1 and 2) and single recombination (1) in 293A cells. Double recombinations (1 and 2) and single recombination (1) between the pAdCR5GFP-L/PS+ and/ or pAdCR5GFP-R/PS+ transfer vector with AdΔPS viral backbone and the possible resulted viruses.

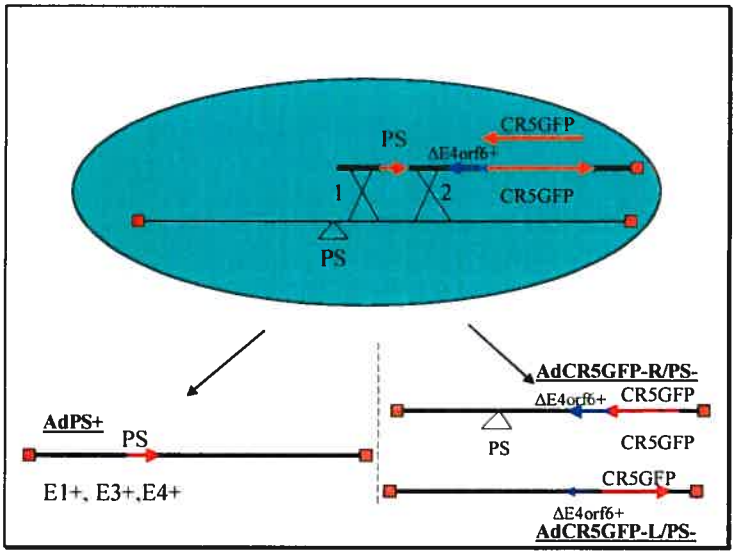


Figure 29: Schema of the possible double recombinations (1 and 2) and single recombination (1) in 293A cells. Double recombinations (1 and 2) and single recombination (1) between the pAdCR5GFP-L/E4$^-$orf6$^+$/PS$^+$ and/ or pAdCR5GFP-R/E4$^-$ orf6$^+$/PS$^+$ transfer vector with AdΔPS viral backbone and the possible resulted viruses.

After the production of the recombinant viruses via positive selection, plaque purification assay was performed to isolate the plaques. After 10- 12 days the recombinant viruses expressing CR5GFP cassette were easily identified by fluorescent microscopy as green plaques on the monolayer culture (see 2.5.2). All the individual plaques were analyzed by PCR for the extracted viral genome for the presence of CR5GFP cassette (results not shown). Among the viruses only the population of AdCR5GFP-R/PS$^-$ was mixed with the Ad5PS$^+$(E3-TK) virus and the purification of the AdCR5GFP-R/PS$^-$ was performed in 293-PS cell line. In our attempt to construct the AdCR5GFP-L/E4$^-$orf6$^+$/PS$^+$(E3-TK) and AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-TK) recombinant viruses, the plaques were perfectly isolated but we did not succeed to purify away the pAdCR5GFP-L/E4$^-$orf6$^+$/PS$^+$ virus in the mixture from the other unwanted recombinant viruses.

The characterisation of the recombinant replicative as well as the control viruses used in this study are described below.

AdCR5GFP-R/PS$^-$

AdCR5GFP-R/PS$^-$ express CR5GFP cassette from a region between RITR and orf1 region of E4 and the orientation of the cassette is toward the right ITR. This virus is a replicative virus in the PS complementing cell lines including 293-PS and it performs a single round of replication in other non-complementary cell lines. This virus was constructed via recombination between the linear pAdCR5GFP-R/PS$^+$ transfer vector and Ad5ΔPS as demonstrated in Figure 28. AdCR5GFP-R/PS$^-$ virus was first selected as a green plaque on 293A cells and the results of PCR for the presence of the CR5GFP cassette on the extracted viral genome showed that it was mixed with the white AdPS$^+$ virus. Several plaque purifications on PS complementing cells (293-PS) were performed to purify the AdCR5GFP-R/PS$^-$ virus. The schema of the AdCR5GFP-R/PS$^-$ is shown in Figure 30.

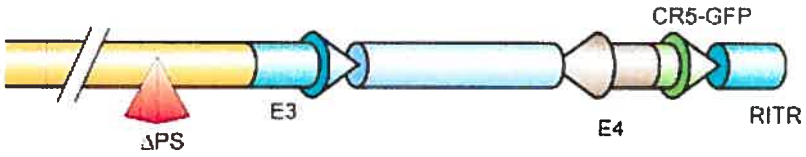


Figure 30: Schema of the AdCR5GFP-R/PS$^-$ recombinant adenovirus

AdCR5GFP-R/PS$^+$

AdCR5GFP-R/PS$^+$ is a replicative AdV expressing CR5GFP cassette from a region between right ITR and orf1 region of E4 and the orientation of the cassette is toward the RITR. This virus was constructed from the AdCR5GFP-R/PS$^-$ virus as a backbone by re-inserting the PS fragment into its original site as previously explained in the material and methods (see 2.5.3). The schema of the AdCR5GFP-R/PS$^+$ is shown in Figure 31.

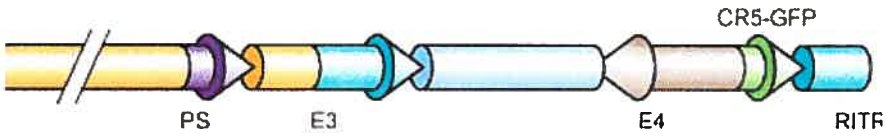


Figure 31: Schema of the AdCR5GFP-R/PS$^+$ recombinant adenovirus

AdCR5GFP-L/PS$^+$(E3-TK)

AdCR5GFP/PS$^+$(E3-tk) is a replicative AdV expressing CR5GFP cassette from a region between RITR and orf1 region of E4 and the orientation of the cassette is toward the left ITR. In this virus the E3 region is deleted and replaced by the PS gene under the control of TK promoter. This virus was constructed via recombination between the linear pAdCR5GFP-L/PS$^+$ transfer vector and Ad5ΔPS as shown in Figure 26. The schema of the AdCR5GFP-L/PS$^+$(E3-TK) is shown in Figure 32.

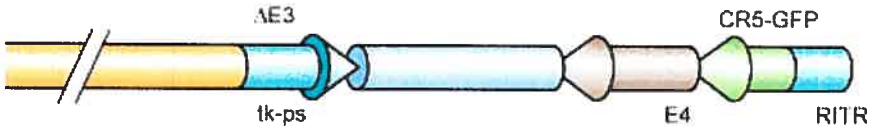


Figure 32: Schema of the AdCR5GFP-L/PS$^+$(E3-TK) recombinant adenovirus

AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-TK)

AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-TK) is a replicative AdV expressing CR5GFP cassette from a region between RITR and orf6 region of E4 and the orientation of the cassette is toward the right ITR. The PS gene is deleted at the original place but the PS gene under the control of TK promoter is placed in the E3 deleted region. The construction of this virus was performed via recombination between the linear pAdCR5GFP-L/E4$^-$orf6$^+$/PS+ transfer vector and Ad5ΔPS as shown in Figure 27. The schema of the AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-TK) is shown in Figure 33.

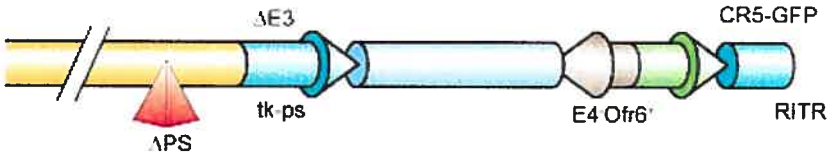


Figure 33: Schema of the AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-TK) recombinant adenovirus

AdCR5GFP-L/E1$^-$

AdCR5GFP/E1$^-$ is a non-replicative AdV expressing CR5GFP from the E1 region and the orientation of the cassette is toward the left ITR. The construction of this virus was described before in material and methods (see 2.5). The schema of the AdCR5GFP-L is shown in Figure 34.

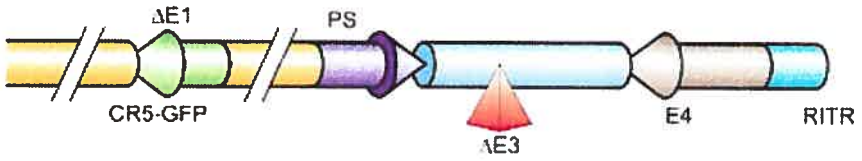


Figure 34: Schema of the AdCR5GFP-L/E1$^-$ recombinant adenovirus

AdCMV5GFP-L/E1$^-$

AdCMV5GFP/E1$^-$ is a non-replicative AdV expressing CMV5GFP from the E1 region and the orientation of the cassette is toward the left ITR. The construction of this virus is described before in material and methods (see 2.5). The schema of the AdCMV5GFP-L/ E1$^-$ is shown in Figure 35.

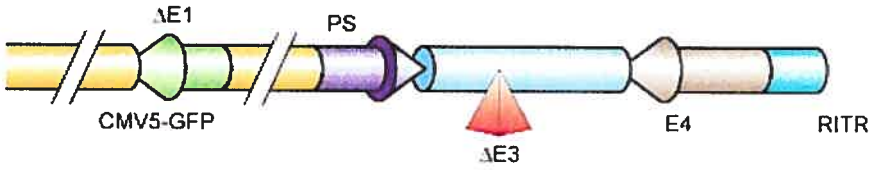


Figure 35: Schema of the AdCMV5GFP-L/E1$^-$ recombinant adenovirus

AdE3$^-$/E4$^-$orf6$^+$

AdE3$^-$/E4$^-$orf6$^+$ is a non-replicative AdV that does not express any transgene. The construction of this virus is described before in material and methods (see 2.5). The schema of the AdE3$^-$/E4$^-$orf6$^+$ is shown in Figure 36.

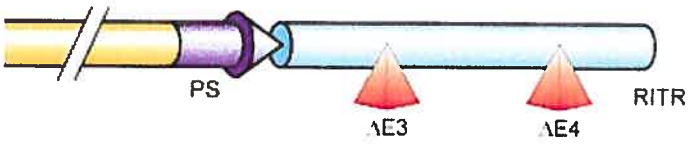


Figure 36: Schema of the AdE3$^-$/E4$^-$orf6$^+$ empty vector

AdPTG3602

The AdPTG3602 is a wild type virus in which all the regions (E1, E3, E4 and PS) are intact (8). The shema of the AdPTG3602 is shown in Figure 37.

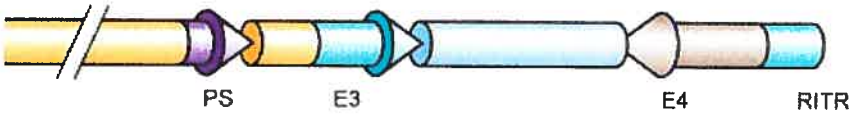


Figure 37: Shema of the AdPTG3602 wild type virus

The summary of the status of different deletions in different regions of viral genome is classified in table III.

virus	E1	PS	E3	E4	Expression cassette-orientation
AdCR5GFP-R/PS$^-$	+	-	+	+	CR5GFP-RITR
AdCR5GFP-R/PS$^+$	+	+ (ori)	+	+	CR5GFP-RITR
AdCR5GFP-L/PS$^+$(E3-tk)	+	+ (E3-tk)	-	+	CR5GFP-RITL
AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk)	+	+ (E3-tk)	-	- (orf 6+)	CR5GFP-LITL
AdCR5GFP-L/E1$^-$	-	+ (ori)	-	+	CR5-GFP-LITR (E1)
AdCMV5GFP-L/E1$^-$	-	+ (ori)	-	+	CMV5-GFP-LITR (E1)
AdE3-/E4$^-$orf6$^+$	+	+ (ori)	-	- (orf 6+)	None
AdPTG3602	+	+ (ori)	+	+	None

Table III: Nomenclature of the AdVs. The AdVs were named on the basis of their transgene and their promoter and other deletions in AdV regions. The abbreviation for the transgene is GFP for Green Fluorecent Protein. The promoters for transgene expression are CMV5-300, a modified Cytomegalovirus immediate-early promoter or CR5, a Cumate inducible promoter. The orientation of the inserted transgenes is shown as R (toward the right ITR) or L (toward the left ITR). The viruses that are PS deleted are shown as PS- and the PS+ viruses are represented for PS in the original position; AdPS+(E1-mlp), PS is in E1 region under the control of mlppromoter or AdPS+(E3-tk), PS is in E3 region under the control of HSK-TK minimal promoter. AdVs with E3 deleted were shown as E3- and those with E4 deletion except for Orf6 are shown as E4-orf6+.

3.9 Analysis of GFP expression from recombinant AdVs in both OFF and ON conditions in the presence or absence of AdcTA in 293Acells

In this experiment in order to have an OFF condition, the 293A cells were infected at MOI of 50 with our recombinant viruses expressing GFP under the control of CR5 promoter, a cumate inducible promoter, and the non-replicative virus expressing GFP under the control of CMV5 promoter, a constitutive promoter. For ON condition all the viruses were co-infected with the AdcTA at MOI of 10 for the transactivator virus and MOI of 50 for the recombinant viruses (Figure 38).

Among all the viruses AdCR5GFP-L/PS+(E3-tk) showed the best induction factor. The mean of GFP expression in ON condition/OFF condition at 24 and 48 hr post infection demonstrated the induction factor of 36 and 44 respectively. The best induction factor is related to the lowest background in off condition as compared to the AdCR5GFP-R/PS+. In all the E3+ AdVs including AdCR5GFP-R/PS$^-$ and AdCR5GFP-R/PS+, the deletion of PS gene in non-complementary cell line (293A) has no significant impact on the level of GFP expression under the ON or OFF conditions. In AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk), which is the only virus with E4 deletion and with the direction of the transgene toward right ITR, the level of background (mean of GFP expression in OFF condition) was increased about 3 times. It seems in this virus with the deletion of E3 and E4 regions, the MLP promoter is situated closer to the transgene and exerts a higher influence on the expression of GFP. By comparing all the recombinant AdVs expressing the CR5GFP from the E4 region with the one expressing the same expression cassette from E1 region, we concluded that all the AdVs with CR5 promoter expressed the GFP in ON condition (presence of AdcTA) better or equivalent to the AdCR5-GFP-L/E1$^-$ that has the same expression cassette in E1 region after 48h. On the other hand the results of comparing the recombinant AdVs expressing the CR5GFP from the E4 region with the one expressing GFP under the control of CMV5 constitutive promoter showed that all the AdVs with the CR5 promoter expressed the GFP at 48hr under ON condition (presence of AdcTA) at the same level or better than the AdCMV5GFP-L/E1$^-$.

The level of mean GFP expression after 48h post infection with AdcTA is remarkably increased in comparison to its expression level after 24h and it could be related to the augmentation in the level of the transactivator by that time. Our results suggest that the insertion of the expression cassettes near the right ITR resulted in transgene expression better than in the E1 region after 48h. Our data showed that in order to generate the replicative recombinant viruses expressing potentially toxic proteins, the pAdE4^{+}/PS^{+}(E3-tk) backbone or pAdE4^{-}orf6^{+}/PS^{+}(E3-tk) in order to increase the transgene capacity with the direction of the transgene toward the left ITR, is the best configuration to have the lowest basal gene expression in OFF condition.

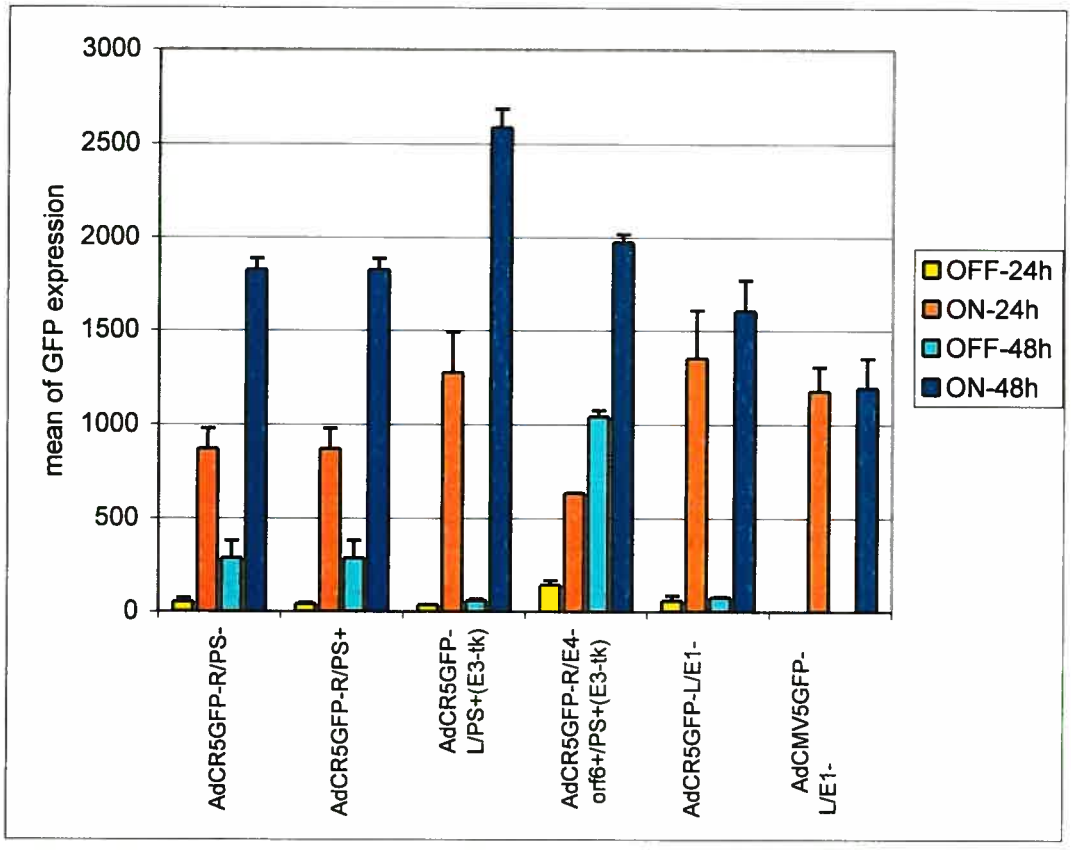


Figure 38: Analysis of GFP expression of the replicative AdVs with CR5 promoter, a cumate inducible promoter, in the presence or absence of AdcTA (an AdV that express cTA protein as a transactivator). 293A cells were transduced with each adV at MOI of 50 (Off condition) or co-infected with AdcTA at a MOI of 10 (On condition). At 24h and 48h post infection, the expression of GFP was measured by flow cytometer. Each experiment was repeated twice and mean of GFP expression were presented as means of two experiment plus the standard deviation).

3.10 Analysis of GFP expression of the recombinant AdVs expressing GFP with CR5 promoter, a cumate inducible promoter, in presence or absence of cumate in Hela-cTA cells

In this experiment HeLa-cTA cells were infected with each of the recombinant replicative AdVs including AdCR5GFP-R/PS$^-$, AdCR5GFP-R/PS$^+$, AdCR5GFP-L/PS$^+$(E3-tk), AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk) in presence (OFF condition) or absence (ON condition) of cumate. The concentration of the cumate was 200 µg/ml. Then the expression of GFP was measured 24h and 48 h post infection by flow cytometry (Figure 39).

The results have demonstrated that deletion of the PS gene in non-complementary cell line (HeLa-cTA) has no significant impact on induction factor (mean of GFP expression in ON condition/ OFF condition). This result, mainly obtained by comparing the AdCR5GFP-R/PS$^-$ and AdCR5GFP-R/PS+ viruses, revealed that their only difference is related to the presence or absence of the PS gene from the original site. By looking at the results of the AdCR5GFP-R/PS+ and AdCR5GFP-L/PS+(E3-tk) viruses we concluded that the orientation of the transgene in E4+/E3+ backbone at 48h post infection did not influence the induction factor. The comparison between the E4+ viruses and E4$^-$orf6$^+$ virus showed that the level of background (mean of GFP expression in OFF condition) increased about 2 times in AdE4$^-$orf6$^+$ after 48h. This part of the results confirmed the conclusion that was obtained from the previous results that the deletion of E3 and E4 regions results in a higher influence of the MLP promoter on the expression of transgene in a configuration toward RITR. Finally the results demonstrated the overall high level of backgroud (mean of GFP expression in OFF condition) for all the viruses as compared to the previous results. We assumed that the problem is related to the leakiness of the CR5/cTA system in this current configuration.

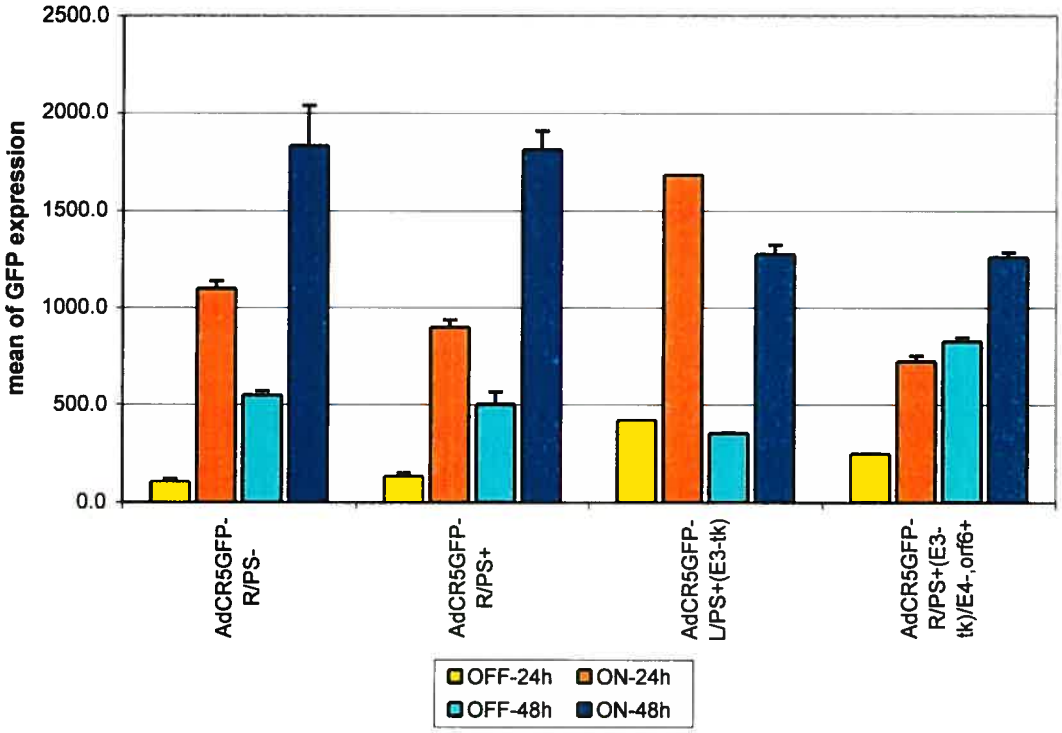


Figure 39: Analysis of GFP expression of AdVs with CR5, a cumate inducible promoter, in presence or absence of cumate from the cTA expressing cell lines (HeLa-cTA). Cells were transduced with each AdV at MOI of 50 in presence (Off condition) or absence (On condition) of cumate. 24h and 48h post-infection, the expression of GFP was measured by flow cytometer. For statistical analysis each experiment was repeated twice and mean of GFP expression were presented as means of two experiments plus the standard deviation.

3.11 Evaluation of PS+ viral progenies expressing GFP under the control of CR5 promoter, a cumate inducible promoter in 293A cells

In this experiment in order to compare the titer (viral progenies) of replicative and non-replicative PS^+ viruses, 293A cells were transduced with each AdVs (AdPTG3602, Ad/E3$^-$E4$^-$orf6$^+$, AdCR5GFP-R/PS$^+$, AdCR5GFP-L/PS$^+$(E3-tk), AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk), AdCR5-GFP-L/E1$^-$ and AdCMV5GFP-L/E1$^-$) at MOI of 50.

At 48h post infection the infected cells were harvested and the virus progeny were measured with a $TCID_{50}$ assay on 293A cells and in 96 wells. In order to have a

reliable result for comparing viral titres, the titer was calculated as a total viral progeny produced by each cell before harvesting the virus (Figure 40).

The results showed that the highest titre is obtained by the wild type adenovirus that has no deletion in viral regions. The titre of the empty vector with the deletions in E3 and E4 regions but orf6$^+$ showed reduction in viral progeny by 3-fold. The reduction of viral progeny could be due to the deletions in E3 and or E4 regions. It has become apparent that each E4 protein independently augments viral DNA replication and late protein synthesis and the production of progeny virions(13). It has been shown that the E3 products are not essential for viral replication *in vitro*. However, in some applications, it might be desirable to retain or even increase the expression of some E3 such as adenovirus death protein (E3-11.6K), which facilitates the release of viral particles from the infected cells(7).

The titer of the replicative AdCR5GFP-R/PS+ showed the reduction of about 4-fold compared to the wild type AdPTG3602. The difference between the titer of two viruses could be related to the insertion of the CR5GFP cassette in the region between the E4 and RITR region. It seems that the insertion of the transgene has oversized the packaging capacity and affected the production of viral progeny. It has been shown that Ad can package up to 2 Kb above its size (38Kb) but this can reduce viral growth (83). The comparison between AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk) and Ad/E3$^-$E4$^-$orf6$^+$empty vector showed a reduction of 2.4 fold in viral progeny. It seems that the deletion in E4 region provided enough space for transgene insertion but the difference in the titer could be related to the suboptimal expression of PS gene under the control of tk promoter in AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk) virus. In addition, the comparison between the AdCR5GFP-L/PS$^+$(E3-tk) and AdCR5GFP-R/PS$^+$ showed the reduction of 8 fold in viral titer. The difference in the titer could be related to the deletion in E3 region and suboptimal expression of PS under the control of TK promoter. Results generated in our laboratory have shown that the suboptimal expression of PS from the TK promoter could affect the viral progeny by 5 fold (Gagnon et al, in preparation).

The comparison between the viruses expressing GFP from E1 region and or from a region between the E4 and RITR under the control of the cumate inducible promoter (CR5) or the constitutive promoter (CMV5) demonstrated that the growth of AdVs was not significantly affected by the insertion of a cassette in the region between E4 and right ITR region.

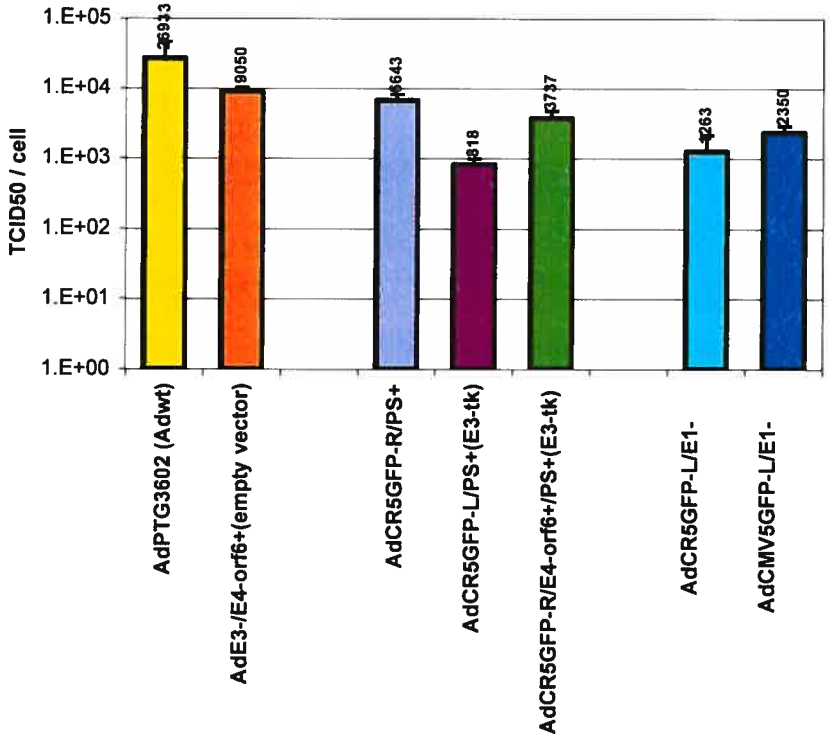


Figure 40: Comparison of PS+ viral progenies of the recombinant replicative adenoviruses expressing GFP under the control of CR5 promoter, a cumate inducible promoter, with the control viruses in 293A cells. Cells were transduced with each AdV at MOI of 50 in triplicate. At 48h post infection the transduced cells were harvested and virus progeny were measured with a $TCID_{50}$ assay on 293A cells in 96 wells plate. The viral titer were presented as $TCID_{50}$ / cell. For statistical analysis each experiment was repeated in triplicate and the data were presented as means of three experiments plus standard deviation.

3.12 Effect of the gene expression on virus progeny

In order to evaluate the effect of the induction of the transgene on the viral progeny the HeLa-cTA cells were transduced with AdCR5GFP-R/E4$^-$orf6+/PS+(E3-tk) and AdCR5GFP-R/PS+ viruses at MOI of 50 in the presence (OFF condition) and absence (ON condition) of cumate (as explained in Figure 40). At 48h post infection, virus progeny was measured with a $TCID_{50}$ assay on 293A cells (Figure 41).

The results showed that although the expression of GFP was 1.5 to 3.6 times higher under the induction condition in HeLa-cTA cells (figure 30) for the AdCR5GFP-R/ E4$^-$orf6+/PS+(E3-tk) viruses and AdCR5GFP-R/PS+, the viral progeny was not reduced. Therefore, we could conclude that the transgene expression did not affect the viral progeny.

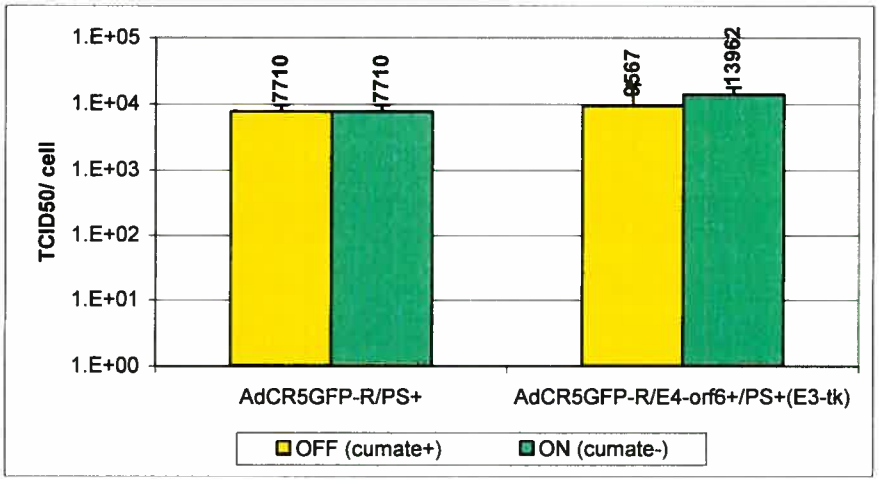


Figure 41: Evaluation of the gene expression in virus progeny. HeLa-cTA cells were transduced in duplicate with AdCR5GFP-R/PS$^+$and AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk) at MOI of 50 in the presence and absence (On condition) of cumate. At 48 h post-infection, virus progeny was measured with a $TCID_{50}$ assay on 293A cells in 96 wells plate. Each experiment was repeated twice and the titer/cell was presented as means of two experiments plus the standard deviation.

Chapter 4 :
Discussion

4.1 Combined suicide gene therapy- with TK and GK

The fundamental goal of this Masters project was based on optimization of the adenovirus vectors in order to have an efficient vector with a strong expression of suicide genes and to to reduce the level of prodrug. One part of the project was to evaluate the activity of combined suicide gene therapy in cancer cells. It has been shown in several publications that using two suicide genes was more beneficial as compared to single suicide gene for cancer therapy. In our laboratory, a study has confirmed the superiority of the combined CD::UPRT (cytosine deaminase:: uracil phosphorybosyl transferase) over CD alone in several tumor cells both in monolayer and spheroid model (84).

In this study we evaluated the cytotoxic activity of AdTK-cite-GK, coexpressing two suicide genes thymidine kinase (TK) from herpes simplex virus 1 and human guanylate kinase (GK) in a dicistronic cassette as compared to AdTK-IRES-GFP, expressing one suicide gene (TK) in a dicistronic cassette with GFP. HSV-TK converts the nucleotide analogs including GCV, into its monophosphate stage via phosphorylation. Once phosphorylated, GCV-P requires further phosphorylation by cellular enzymes to produce its toxic form, which terminats DNA synthesis. GK can carry out both subsequent phosphorylation steps and has been reported to be present in many tissue extracts. It has been demonstrated in a recent publication that co-expression of GK with TK in noncancerous cells enhances prodrug cell killing *in vitro* and suppresses vascular smooth muscle cell proliferation *in vivo* leading to administration of GCV at lower doses (75). It has been shown that the high doses of GCV are associated with side effect such as nephrotoxicity and bone marrow supression (75).

In our study we evaluated the coexpression of GK with TK to test whether the coexpression of GK with TK might enhance phosphorylation of GCV in cancer cells. In order to accomplish this objective, the cytotoxic activity of AdTK-IRES-GFP and AdTK-cite-GK viruses were characterised in two cancerous cell lines: U87 and TOV

21G. The concentration of GCV was measured at IC_{50} in order to compare the cytotoxic activity of both viruses (Figures 17 and 18). The results demonstrated that the coexpression of GK with TK did not improve the cytotoxic effect of TK in cancer cells. In order to confirm the results obtained in cancer cells, we evaluated the cytotoxic activity of our viruses in normal cells. We used the Huvec cells, which are very similar to the cells that were used in the previously publication (75) in order to see whether we could get the same results as them (Figure 20). MRC-5 cells were used as another normal cell line (Figure 19). The results of the cytotoxic activity of our viruses in normal cells confirmed the results of the cancerous cells that the GK gene has no superior effect on cytotoxic effect of TK. The expression of TK and GK and the enzymatic activity of GK were evaluated in order to support our conclusion. The expression of the TK gene from AdTK-IRES-GFP and AdTK-cite-GK in four different cell lines were evaluated and showed the same level of expression from each cell line (Figure 21). The expression of GK gene and also the enzymatic activity of GK were characterised and showed that the GK gene is perfectly expressed and is enzymatically active (Figures 16 and 22).

At the end we compared our results with the data shown in abovementioned publication that demonstrated the improvement in cytotoxic activity with coexpression of TK and GK in vascular smooth muscle cells (75). In this publication the coexpression of GK with TK in comparison to the TK alone was shown as a result of a transfection experiment. They have shown that the transfection of vascular cells with TK-cite-GK (pAdEF1αTK-cite-GK) could increase cell killing by 2-fold as compared to TK alone (pAdEF1α-TK) at a dose of 0.5 μM GCV. They then compared the cell viability of the infected vascular cells with three different TK-cite-GK expressing viruses under the control of three different promoters. They evaluated the effect of the coexpression of GK with TK via different promoters on reducing the level of the prodrugs ganciclovir (GCV) and acyclovir (ACV). They never confirmed the results of transfection with their constructed viruses to test whether the same difference in cell killing was obtained via coexpression of TK and GK compared to TK alone was reproducible. Since the results of transfection could vary from one transfection to another due to the quantity of DNA,

the results of transfection need to be confirmed via transduction, we can raise doubt about the validity of their results.

4.2 Correlation between the increasing of TK and the bystander effect in U87 cells

Until very recently gene therapy either developed AdVs expressing suicide genes or oncolytic AdVs. Because of their efficacies, new AdVs are now combining oncolysis and suicide gene therapy (85;86). Since the coexpression of two suicide genes including TK and GK did not show a significant improvement in cytotoxic activity as compared to TK alone in several cancerous and noncancerous cells, in this part of study we evaluated the relation between the increasing amount of suicide gene (TK) and the cytotoxic activity in cancer cells. The objective was to increase the viral genome carrying the suicide gene either through the increase of MOI or through the replication and to test the consequences on cytotoxic activity and bystander effect.

Our data have shown that increasing copy numbers of the suicide gene (TK) via increasing the MOI of AdTK-IRES-GFP virus from 20 to 200 in U87 cells has no improved on the bystander effect in presence of GCV (Figure 23). It has been shown in a publication that there is a proportional increase in transgene expression and the MOI (up to MOI of 1000) in permissive and non-permissive cells infected with AdCMV5-GFPq virus (78). The results of the western blot demonstrated that there is a correlation between the increase in MOI of AdTK-IRES-GFP and the TK expression (results not shown). We showed that there is about 10- fold difference for TK expression between the MOI of 20 and 200, which confirmed the results of abovementioned article. On the other hand we tried to amplify the AdTK-IRES-GFP genome through replication by co-infecting it with an E1A+ virus at MOI of 20. The results shown in Figure 24 revealed that amplifying the AdTK-IRES-GFP did not improve the bystander effect as compared to the nonreplicative AdTK-IRES-GFP virus. It seems that the correlation between the high level of TK expression and the bystander assay in cancer cells is affected by different factors. As already discussed in the introduction section a primary major

limitation of the bystander effect in HSV-TK/GCV system has been attributed to gap junction dependence, involving the passage of cytotoxic phosphorylated GCV derivatives between the cells.

On the other hand we evaluated the effect of the GCV on the replication of AdTK-IRES-GFP in 293A cells. Our data showed the decrease in viral progeny by increasing the concentration of GCV. Our results confirmed the virostatic effect of GCV as shown by another group (82). This leads us to believe that the combination of HSV-TK suicide gene and oncolysis may not result in much improvement and is may provide as another limitation for bystander effect in replicative/dissiminative platform.

4.3 Introducing an effective configuration for cloning between E4 and RITR region

Although replication of AdV demonstrated no benefit to suicide gene therapy with HSV-tk in our platform, results generated in our laboratory have shown that it is favorable with CU suicide gene (Bourbeau et al., in preparation). In an attempt to develop a replicative adenovirus expressing CD::UPRT suicide gene the E1A gene of adenovirus was introduced downstream of the transgene, following an internal ribosomal entry site (IRES) and this expression cassette replaced the E1 region of adenovirus (Bourbeau et al., in preparation). The presence of E1A allowed the replication of the viral genome and the expression of viral proteins. But this E1A+ virus showed difficulty for viral growth and production of viral progeny compared to the wild type virus. Based on the other publications this alteration in viral growth is related to the absence of E1B 55KDa gene. It has been shown that E1B55KDa- deleted mutants replicate significantly less well than Ad5WT (87). Responsible for this phenotype is most likely the lacking ability of the E1B 55KDa gene product to relieve the restrictions on adenoviral replication established by the cell cycle (88) and to facilitate the transportation of viral mRNA during the late stage of a lytic adenoviral infection (89).

In another part of our study, we have explored another approach to introduce a more efficient replicative AdV in order to overcome the limitations of the current replicative AdVs. We tried to introduce the transgene in a region other than E1, which was between the E4 region and the RITR. It has been shown by other groups that in order to develop a tissue specific transgene expression, placing the promoter in close proximity to the E1A enhancer results in several fold increase in transgene expression in undesired tissues as compared to the cloning site near the right ITR (90). In this study we determined the best strategy for cloning the transgene (GFP) under the control of cumate regulatory promoter (CR5) in a region between E4 and RITR. We tried to find a configuration with the highest transactivation in presence of the transactivator and the lowest background in the absence of the transactivator. We used two different adenoviral vectors, one E4 completed and the other E4 deleted and orf6 added in order to have about 2.5Kb extra space for the transgene insertion. In this study we analysed the GFP expression of the replicative viruses in 293A cells co-transfected with AdcTA as well as in Hela-cTA cells that stably expresses cTA protein (Figures 38 and 39). Our data have shown that the the cloning of the CR5GFP transgene in a region between E4 and RITR with the orientation toward the left ITR gives the best induction factor with the minimal backgroung (off condition). We demonstrated that the cloning of the CR5GFP toward the right ITR in an E3 and E4 deleted backbone had the highest GFP expression as a background (off condition). It seems that in the E3 and E4 deleted platform MLP promoter, which is situated closer to the transgene could strongly enhance the GFP expression. We observed that in 293 A cells the level of GFP background in the absence of cTA protein (in the absence of cotransfection by cTA virus) is increased after 48h post infection. The same results were obtained from Hela-cTA cells in presence of cumate (OFF condition) and these results are related to the leaky expression of the transgene from CR5 regulatory promoter in the current platform.

In order to determine whether the transgene expression has any effect on viral progeny, we tested the effect of induction of the transgene expression on two different replicative viral progeny in Hela-cTA cells in the off and on conditions. The results

showed that the overexpression of the transgene has no disadvantageous effect on viral growth (Figure 41). In addition, we evaluated the titre of our replicative viral progenies and the first generation AdVs and the wild type virus in 293A cells as shown in Figure 40. The results of the production of our replicative viral progenies showed that there are remarkable difference between the titre of our replicative viruses and the first generation AdVs. We could conclude that the presence of the transgene in the region between E4 and RITR instead of the E1 region had no dilaterious effect on viral progeny. It could be suggested based on our results that there are different factors involved in decreasing the viral progeny of our replicative viruses as compared to the Ad wild type. Deletion in E3 region (7), suboptimal expression of PS under the TK promoter (Gagnon et al., in preparation) and oversizing viral genome (83) are the main factors. It has been shown that the maximum length of inserts that can be introduced in AdVs is limited due to a tightly controled packaging capacity of the adenovirus capsid. AdV has a theoretical packaging limit of 38 Kb, which is 2Kb above its normal size. A study was done with replication deficient adenoviruses containing expression cassettes of various sizes to yield oversized AdVs from 106.5% to 108.6% of viral genome (83). Their results showed an unusually low percentage of recombinants following transfection as well as considerable genetic instability during amplification and plaque purification. Based on the publications it is recommended not to oversize the packaging capacity.

4.4 Optimization of the AdV vectors for cancer gene therapy

We could extend our findings to design a potential replicative/nondissiminative adenovirus for application in cancer therapy. It has been shown that the delta protease platform was developed as a tool for applications where high level of transgene is required (e.g. cancer, vaccination, recombinant protein production, etc) (8). In order to produce the replicative /nondissiminative AdV expressing the transgene from a region between E4 and RITR a standard method should be developed via redesigning the Adeasy system. In this method the transfer vector could be modified to enable us to introduce the transgene to the region between the E4 and RITR instead of the E1 region

and the recombination could occur between the PS deleted Adeasy and the shuttle vector.

We would design a platform to introduce the gap junction free suicide genes (eg. CD, CD::UPRT) under the control of a tumor specific promoter (eg. CXCR4, EGP-2, Cox-2 and others). Tumor specific promoters are employed to restrict transgene expression or viral replication in an effective way in order to increase specificity towards tumor tissues and to reduce adverse effects in non-target tissues. In this design the desired tumor specific promoter is followed by the cumate operator sequence (CuO) downstream of the promoter. The CuO operator would allow us to down regulate the expression of the toxic suicide gene at the step of viral production in 293CymR cells as was successfully done for production of AdTK-IRES-GFP and AdTK-cite-GK viruses. This cassette is introduced in a region between the E4 and RITR with the orientation toward the left ITR. Our data showed that in our replicative virus platform the deletion in the E3 and E4 (but orf6 added) regions gives a space of 3.1 Kb and 2.5 Kb respectively. These deletions would allow us to insert a cassette of about 5.5Kb. Although it has been shown in several publications that the tumor specific promoters has been very powerful in delivering toxic genes to tumors, the efficacy of our designed platform should be tested in vivo.

4.5 Conclusion

In conclusion, in our attempt to develop a new strategy for cancer therapy using combined suicide gene therapy, we have determined that the better phosphorylation of GCV via co-expression of GK with TK was not a rate- limiting step in tumor cells including U87 and TOV 21G cells. We determined that the overexpression of TK suicide gene in U87 cells either by increasing MOI or through replication did not improve the cytotoxic activity and the bystander effect.

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ANNEXE I: Plasmids and viruses used in this study

List of plasmids	source
pAdenoVator-CMV5(CuO)TK-IRES-GFP	work done in this thesis, unpublished
pAdenoVator–CMV5(CuO)-IRES-GFP	Q-biogen, Montreal, Canada
pGT60codAupp	InvivoGen, San Diego
pBS-TKciteGK vector	reference 74
pAdenoVator-CMV5(CuO)mcs	Q-biogen, Montreal, Canada
AdenoVator-CMV5(CuO)TK-Cite-GK	work done in this thesis, unpublished
pAdE4CR5GFP-R/PS$^+$	work done in this thesis, unpublished
pAdE4CR5GFP-L/PS$^+$	work done in this thesis, unpublished
pAdE4ext	work done in our lanoratory, unpublished
pAdCR5GFP	work done in this thesis, unpublished
pAdCR5GFP-R	work done in this thesis, unpublished
pAdCR5GFP-L	work done in this thesis, unpublished
pAdCMV5DCGFPq/TK-PS	Gagnon et al, in preparation
pAdE4$^-$orf6$^+$CR5GFP-R/PS$^+$	work done in this thesis, unpublished
pAd E4$^-$orf6$^+$CR5GFP-L/PS$^+$	work done in this thesis, unpublished
pCymR/tk-neo	reference 33
pHSE*PSPAV3-DC-GFPq/tk*Hygro	Castagner., 2002 unpublished

List of viruses	source
AdCMV5(CuO)TK-IRES-GFP	work done in this thesis, unpublished
AdCMV5(CuO)TK-cite-GK	work done in this thesis, unpublished
Ad(PS$^+$)CU-IRES-E1A	Bourbeau et al., in preparation
AdCR5GFP-R/PS$^+$	work done in this thesis, unpublished
AdCR5GFP-L/PS$^+$(E3-tk)	work done in this thesis, unpublished
AdCR5GFP-R/E4$^-$orf6$^+$/PS$^+$(E3-tk)	work done in this thesis, unpublished
AdCR5GFP-R/PS$^-$	work done in this thesis, unpublished
AdCR5GFP-L/E1$^-$	reference 77
AdCMV5GFP-L/E1$^-$	reference 33
AdE3-/E4$^-$orf6$^+$(empty vector)	Elahi et al, in preparation
AdPTG3602 (Adwt)	Oualikene et al., 2000
Ad5ΔPS	Oualikene et al., 2000
AdcTA	reference 33
AdCMV5-Cuo-LacZ	reference 33