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TIINA WAHLFORS

Enhancement of HSV-TK/GCV suicide gene therapy of cancer

Doctoral dissertation

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ABSTRACT

Gene therapy has become a promising alternative treatment form for cancer. Among the broad range of different genetic means to reduce the tumor growth, herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) suicide gene therapy regimen is the best known approach. In this type of therapy, cancer cells are manipulated to express HSV-TK, followed by administration of the prodrug, the antiviral drug GCV. This prodrug is relatively harmless to normal cells but efficiently kills cells that express HSV-TK. The HSV-TK/GCV suicide gene therapy has been tested extensively, in the laboratory and some recent clinical results have also demonstrated the potential of this treatment form. However, cancer patients still cannot be cured with the method, indicating that this approach needs refinement before true clinical success can be achieved.

One way to enhance suicide gene therapy is to increase the number of S phase cells in the tumor, since these cells are undergoing DNA replication and are thus vulnerable to the toxic form of GCV. It is known that alpha-difluoromethylornithine (DFMO), a well-known and well-tolerated polyamine biosynthesis inhibitor, can generate a prominent cell cycle arrest and when DFMO is withdrawn from the cells, they begin to divide again and display an elevated proportion of S phase cells for a certain period of time. This window of increased S phases may be exploited for enhancement of HSV-TK/GCV suicide gene therapy, at least in theory. To verify this hypothesis, the novel combination of polyamine depletion and suicide gene therapy was tested first in cultured cells and thereafter in a mouse tumor model. Furthermore, other types of drugs affecting the polyamine homeostasis or the cell cycle were investigated for this enhancing effect.

The combination of HSV-TK/GCV gene therapy and DFMO resulted in an enhanced cytotoxic effect in cultured rat and human tumor cells. This synergistic effect was achieved only when the timing between DFMO treatment and HSV-TK/GCV gene therapy was optimal. A similar effect was also observed in a subcutaneous mouse tumor model, demonstrating the efficacy of this combination *in vivo*. However, other attemps to manipulate the polyamine homeostasis or cell cycle phase distribution (polyamine catabolism activation with N^1,N^{11}-diethylnorspermine, serum deprivation or treatment with aphidicolin, hydroxyurea, lovastatin, mimosin and resveratrol) did not significantly enhance the efficacy of HSV-TK/GCV gene therapy. Moreover, the duration of the S phase effect needs to be long enough to allow enhancement of HSV-TK/GCV –mediated cell killing.

In conclusion, our results indicate that correctly timed polyamine depletion with DFMO is an efficient way to enhance the HSV-TK/GCV gene therapy approach in human tumor cells and the effect also appears to be achievable in animal tumor models. Since both DFMO and HSV-TK/GCV gene therapy have been extensively tested in clinical trials and their safety profiles have turned out to be excellent, it is realistic to hope that this combination treatment will be successful in clinical studies. However, only further preclinical studies with more relevant (orthotopic) animal models and primary human tumor material will reveal the true utility of the combination.

National Library of Medicine Classification: QZ 266, QZ 52, QU 141, QU 61, QU 375
Medical Subject Headings: neoplasms/therapy; gene therapy; genes, transgenic, suicide; cell death; simplexvirus; thymidine kinase; ganciclovir; cell cycle; S phase; polyamines/antagonists & inhibitors; eflornithine; cells, cultured; disease models, animal

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ABBREVIATIONS

AAV	adeno associated virus
ACV	acyclovir
AdoMetDC	S-adenosyl-L-methionine decarboxylase
ANOVA	analysis of variance
APC	adenomatous polyposis coli
ATCC	American type culture collection
Bcl-2	B-cell lymphoma
cAMP	cyclic adenosine monophosphate
CD	cytosine deaminase
COX-2	cyclooxygenase enzyme, subtype 2
CPA	cyclophosphamide
CYP2B1	cytochrome p450
DENSPM	N^1,N^{11}-diethylnorspermine
DFMO	difluoromethylornithine
eIF5A	eukaryotic initiation factor 5A
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorter
FADD	Fas-associated death domain protein
GCV	ganciclovir
GCVTP	ganciclovir triphosphate
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
HPLC	high-performance liquid chromatography
HSV-TK	herpes simplex virus type I thymidine kinase
i.p.	intra peritoneal
IKBA	interleucine kappa B alpha
JCRB	Japanise collection of research bioresources
MAPK	mitogen-activated protein kinase
MAT	multifocal angiostatic therapy
mDNA	mitochondrial DNA
MCP-1	monocyte chemoattractant protein-1
MGBG	methylglyoxyl bis(guanylhydrazone)
NF-kB	nuclear factor-kappa B
NMR	nuclear magnetic resonance
ODC	L-ornithine decarboxylase
PAO	polyamine oxidase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
PGE2	prostaglandin E2
PI	propidium iodide
Put	putrescine
SiRNA	short interfering RNA
SMO	spermine oxidase
Spd	spermidine
Spm	spermine
SSAT	spermidine/(spermine) N^1-acetyltransferase
TNF-α	tumor necrosis factor alpha

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by the corresponding Roman numerals.

I Pasanen T., Karppinen A., Alhonen L., Jänne J. and Wahlfors J. Polyamine biosynthesis inhibition enhances HSV-1 thymidine kinase/ganciclovir-mediated cytotoxicity in tumor cells. Int J Cancer (2003) *104*, 380-388

II Pasanen T., Hakkarainen T., Timonen P., Parkkinen J., Tenhunen A., Loimas S. and Wahlfors J. TK-GFP fusion gene virus vectors as tools for studying the features of HSV-TK/ganciclovir cancer gene therapy *in vivo*. Int J Mol Med (2003) *12*, 525-531

III Wahlfors T., Hakkarainen T., Jänne J., Alhonen L., and Wahlfors J. *In vivo* enhancement of Herpes simplex virus thymidine kinase/ganciclovir cancer gene therapy with polyamine biosynthesis inhibition. Int J Cancer, in press.

IV Wahlfors T., Karppinen A., Jänne J., Alhonen L. and Wahlfors J. Polyamine depletion and cell cycle manipulation in combination with HSV thymidine kinase/ganciclovir cancer gene therapy. Int J Oncol, in press.

CONTENTS

ORIGINAL PUBLICATIONS I-IV

1 INTRODUCTION

Progress in biology, biochemistry and medicine has had an enormous impact on the development in the modern world. For example, the roles of selective breeding of house animals and plants, vaccination and antibiotics have been crucial for the establishment of civilization as we know it today. In the field of modern medicine, gene therapy is one of the most publicized and also most controversial areas but it does hold the promise of becoming one of the major treatment regimens in the future. Gene therapy holds immense potential to combat genetic disorders as well as acquired diseases such as cardiovascular disorders and cancer. Indeed, the vast majority of current gene therapy trials are anti-cancer therapies, despite the fact that the initial purpose of gene therapy was to treat monogenic diseases. That is understandable, since more than 10 million people each year become affected with one of the numerous life-threatening cancers, whereas inherited monogenic diseases are rare and concern only a very small number of people.

The frequent incidence of cancers, the lack of efficacy of the present oncological treatment forms and particularly the diverse genetic background of different malignant diseases has led to creation of a variety of gene therapy approaches to combat these diseases. The devastating impact of cancer cells has been restricted with restoration of normal cell function by introducing wild type tumor suppressor genes or oncogenes into the cancer cells. Inhibition of vascularisation of tumors as well as boosting the immune response against cancer can also be exploited. Furthermore, anticancer treatments can also employ suicide gene therapy strategies. In these approaches, a suicide gene is delivered with the aid of a vector into the cancer cells. Transduced cells then become vulnerable to a non-toxic prodrug and are destroyed.

Suicide gene therapy benefits from a phenomenon called the bystander effect which mitigates the suboptimal transfection efficiency obtained with current gene transfer methods. As a consequence of low transduction efficacy, the best results with suicide gene therapy have been achieved when it is combined with other treatment forms i.e. surgery or radiation. To enhance the efficacy of suicide gene therapy, novel approaches, for example mutated the use of suicide genes with enhanced enzymatic activity or new formulations of prodrugs with higher affinity for the suicide protein have been tested. Also, enhancement of the bystander effect that may sometimes be mediated via gap junctions has been attempted by introducing a gap junction - forming protein connexin into tumor cells along with a suicide gene. Combinations of two suicide gene systems have also observed to increase the cytotoxic effect of prodrug regimens.

In this study, we tested a combination of traditional chemotherapy and suicide gene therapy in a novel way. Our results show that the cytotoxic effect of herpes simplex virus thymidine kinase with the prodrug ganciclovir can be enhanced *in vitro* and *in vivo* with polyamine biosynthesis inhibition in conjunction with cell cycle alterations.

2 REVIEW OF THE LITERATURE

2.1 GENE THERAPY

2.1.1 OVERVIEW

The term genetic manipulation is used when genetic material is transported into the host organism's genome. In gene therapy approaches, genetic material is transferred in order to cure diseases (Morgan and Anderson, 1993). This form of therapy is considered to be one of the most promising future treatment forms. It was originally developed for genetic diseases where a single gene is functionally defected. The idea was to introduce a functionally normal gene into the host genome to compensate for the consequences of the mutation. This original concept has become expanded and nowadays gene therapy signifies any approach using genetic material to prevent or treat a variety of diseases, including multifactorial and somatic genetic diseases, such as cancer (Barzon et al., 2004).

The possibility of the utility of DNA as therapeutic agent was discussed already in the early 70's, when the ability of pseudoviruses to deliver genes was discovered (Osterman et al., 1970; Qasba and Aposhian, 1971). The first gene transfer into humans was done in 1971 by Stanfield Rogers and it was made without any official license (reviewed in Friedmann, 2001). His actions were judged as unethical and even dangerous by the other scientists. In addition to the critical and ethical discussion about gene therapy, a lot of preliminary studies were conducted in 80's. Furthermore, another unauthorized study with human patients was done by a respected biomedical scientist Martin Cline. He attempted to treat two patients with severe β-thalassemia by transfecting bone marrow cells with recombinant human β-globin gene (reviewed in Beutler, 2001). The patients were neither cured nor harmed but Dr. Cline was forced to resign his department chairmanship and lost several research grants (Sun, 1981). However, the positive results from cell culture experiments and animal studies eventually led to the first approved gene therapy treatment trial in 1990. The disease in this trial was a form of severe combined immunodeficiency (SCID), which is a consequence of adenosine deaminase (ADA) deficiency. The patients suffer from a weakened immune system and are thus vulnerable to life-threatening infections. The first SCID patient in this trial was four year old Ashanti Desilva, whose T-cells were collected and delivered back after new genes had been introduced into them. The therapy did not achieve a complete cure, but it lowered the amount of drug needed for treating the disease (PEG-ADA, costing more than 100,000 $ a year) (Blaese et al., 1995).

The recent progress of molecular biology and medicine in 90's, has helped researchers working on gene therapy to develop better and safer vectors for gene transfer and increased the understanding of many diseases. Finally, in 2000, the first patients were cured with the aid of gene therapy. These patients were children with X chromosome linked severe combined immunodeficiency (X-SCID) (Cavazzana-Calvo et al., 2000). Unfortunately, three out of the eleven patients had few years later developed abnormal white blood cell growth due to retroviral vector integration into the LMO2 region in chromosome 11p13 (Hacein-Bey-Abina et al., 2003). This may have lead to activation of proto-oncogene in T- cells causing a leukemia -like syndrome (Kohn et al., 2003). Also cancer has now successfully been treated with gene therapy. Glioblastoma has been one of the most extensively studied cancers in the context of gene therapy trials. Increased survival times have been achieved from randomized controlled studies with suicide gene therapy approaches (Immonen et al., 2004; Sandmair et al., 2000).

2.1.2 GENE THERAPY IN CLINICAL USE

Over the past decade, the focus of gene therapy research has moved increasingly from pre-clinical experiments to clinical trials. Before one can treat patients with an experimental procedure, there are a number of regulatory and institutional procedures that have to be carried out. In the case of gene therapy, biosafety aspects have to be dealt with and issues related to the vector safety need to be carefully evaluated. Before approval of a clinical trial, the therapeutic agent has to be thoroughly tested for its efficacy *in vitro* and *in vivo*. Furthermore, toxicity and biodistribution studies have to be performed in an appropriate animal model. Clinical trials are categorized from phase I to III, starting from nonrandomized safety studies with low a number of patients (phase I), followed by somewhat larger efficacy studies that also aim at determining the limiting toxic dose of the vector (phase II). Finally a randomized, placebo-controlled study with a large number of patients is conducted to determine the clinical benefit of the therapy (Hermiston and Kirn, 2005). After passing all these phases, the first gene therapy protocol was approved for clinical practice in 2003 in China (Pearson et al., 2004). This first commercial cancer gene therapy regimen utilizes an adenoviral vector with p53 and it is aimed against head and neck squamous cell carcinoma. Thus, the first gene medicine is already commercially available and, not surprisingly, it is an anti-cancer agent. However, a number of different trials utilizing genetic material have been conducted during the last two decades. **Table 1.** summarizes some examples of the diseases that have been targeted in clinical gene therapy trials.

Table 1. Examples of clinical trial in gene therapy research.

Target disease	Delivered gene	Phase of clinical development	References
Inherited disorders			
Hemophilia	FIX or FVIII	I	(Kay et al., 2000; Powell et al., 2003)
Cystic fibrosis	CFTR	I	(Alton et al., 1999)
Chronic granulomatous disease	$p47^{phox}$	I	(Malech et al., 1997)
Acquired diseases			
Cancer			
head and neck squamous cell carcinoma	p53	Approved	(Pearson et al., 2004; Peng, 2005)
glioma	HSV-TK	I/II	(Immonen et al., 2004)
Alzheimer's disease	NGF	I	(Tuszynski et al., 2005)
Lower limb ischemia	VEGF	II	(Mäkinen et al., 2002)
Infectious diseases			
HIV-1 infection		I-III	http://www.wiley.co.uk/genmed/clinical/
Hepatitis virus infection		I	http://www.wiley.co.uk/genmed/clinical/

CFTR; Cystic fibrosis transmembrane conductance regulator, FIX and FVIII; clotting factors, HSV-TK; herpes simplex virus thymidine kinase, NGF; nerve growth factor, p47; regulatory protein, p53; tumor suppressor protein, VEGF; vascular endothelial growth factor

2.1.3 VECTORS AND GENE DELIVERY SYSTEMS

To achieve true clinical success, gene therapy has to overcome several major barriers. One critical improvement is the need to develop better gene delivery tools, since the current methods are usually insufficient for most treatment purposes. There are three desired features for optimal vectors i.e. 1) ability to transduce cells of different tissues, 2) the possibility to target the vectors to a certain tissue, 3) a stable, sufficiently long-lasting and regulated transgene expression in the target tissue. Side effects caused by gene transfer vectors, such as a hazardous interaction with the vector and the host genome, or the appearance of an immunological reaction against the therapeutic gene or vectors are problems that are actively being investigated. One further hurdle to be overcome in vector development is the inefficient manufacturing methods for high titer vectors. High titers of virus vectors are needed to obtain a reasonable transgene expression for a true clinical benefit in gene therapy trials. These examples of the problems in vector development illustrate the need for creative vector design to enhance the efficacy and safety of therapeutic gene transfer (Spink and Geddes, 2004).

There are two main groups of gene transfer vehicles: viral and non-viral vectors. Viruses have been designed by evolution that has turned them into gene delivery machines whose only goal is to transfer genetic material into the host cell and multiply. The fundamental idea of turning the wild type viruses into gene transfer vehicles involves verification of the components needed for replication, the assembly of viral particles, the packaging of viral genome and the delivery of transgene. Dispensable genes are deleted to ensure that the virus is replication-defective and less immunogenic. The transgene is then inserted into the vector construct together with transcriptional regulatory elements. In vector production, genes for replication and virion components are delivered to producer cells together with a vector construct in order to make recombinant viruses (Verma, 2005). A broad range of different viruses has been utilized in gene therapy protocols. For example, adenoviruses, retroviruses, lentiviruses and herpes viruses have been tested in a wide variety of applications (Table 1).

Identification of molecular defects associated with cancer has made it possible to design vectors that can selectively replicate in tumor cells and result in death of malignant cells i.e. oncolysis. These replication-selective viruses increase tumor transduction efficiency and also help the possible therapeutic agent to spread all over the target tissue (Biederer et al., 2002). However the oncolysis itself is the primary reason for therapeutic response and few of these vectors contain additional transgene.

The non-viral gene delivery systems offer significantly less toxic alternatives for gene transfer compared to the viral vectors, but their efficiency is usually lower (Djurovic et al., 2004; Hagstrom et al., 2004). However, the low immunogenicity of non-viral methods makes it possible to carry out repeated vector administrations, which can, to some extent, compensate for the poor gene transfer efficacy (Lundstrom and Boulikas, 2003). Furthermore, the unlimited transgene capacity and simple manufacturing production are considered to be advantages of non-viral methods (Gardlik et al., 2005). Intramuscular injection and gene gun mediated transfer of naked DNA has shown promising results in clinical trials of cytokine gene therapy against cancer (Nishitani et al., 2000). Instead of naked DNA administration, artificial vectors have been developed to improve the penetration of DNA into the cells. Cationic liposomes, formed by different types of lipids, protect the DNA from degradation and facilitate penetration into the host cell via the endocytosis (Zhdanov et al., 2002). Cationic liposomes have been used for example in a human brain tumor trial (Yoshida et al., 2004). Cellular gene delivery, i.e. using genetically modified cells as therapeutic vehicles, is also gaining attention and may be one realistic choice for treatment in the future. Promising data from animal experiments has been

achieved with stem cells derived from different sources (Brown et al., 2003; Lee et al., 2003; Moore et al., 2004; Nakamura et al., 2004). One rather original idea was also to utilize the DNA condensing properties of polyamines and use lipopolyamines as nucleic-acid carrier (Ahmed et al., 2005; Blagbrough et al., 2003). **Table 2**. summarizes the features of the most commonly used vector types in gene therapy research.

Table 2. Main gene delivery systems used for gene therapy.

Vectors	Genetic material	Packaging capacity	Integration	Main Advantages	Main disadvantages
Retrovirus	RNA	8 kb	Yes	enable long expression, pseudotyping increases host cell tropism, low toxicity	inability to infect non-dividing cells, potential insertional mutagenesis
Lentivirus	RNA	8 kb	Yes	infection of non dividing cells, broad tropism	safety concerns since many of them are based on human immunodeficiency virus, potential for insertional mutagenesis
Herpes virus	dsDNA	40 kb	No	large packaging capacity, strong tropism for neurons, oncolytic strains available	highly immunogenic, transient transgene expression in cells other than neurons
Adenovirus	dsDNA	10 kb	No	high titers, oncolytic strains available	highly immunogenic, transient expression
AAV	ssDNA	<5 kb	No	ssDNA viruses, broad tropism, integration, low packaging capacity	low transgene capacity
Liposomes	-	unlimited	No	easy to produce, low immunogenicity	inefficient gene delivery *in vivo*
Stem cells	-		No	low immunogenicity	*ex vivo* transduction

2.2 CANCER GENE THERAPY

Cancer is a genetic disease where the malignant cells contain somatic mutations in their growth and death associated genes. Mutations in cancer cells promote their ability to divide in an uncontrolled manner and furthermore allow these cells to invade and metastasize to surrounding tissues. The better understanding of molecular biology of cancer has made it possible to treat cancer on the basis of its molecular characteristics (Gottesman, 2003). This has been successfully utilized in gene therapy of malignancies: according to the Journal of Gene Medicine Database (The Journal of Gene Medicine; http://www.wiley.co.uk/genmed/clinical/), of all gene therapy clinical trials 66.4% are aimed against cancer (**Figure 1**.).

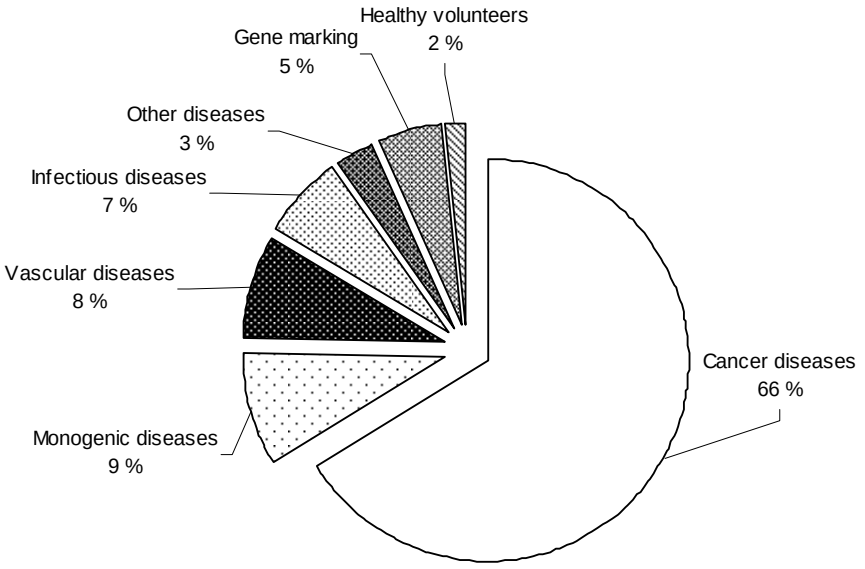


Figure 1. Gene therapy clinical trials conducted world-wide as of 2005 (n = 1076).

Cancer gene therapy research is focusing on three major themes, 1) to discover new means for killing or slowing down the growth of cancer cells, 2) the improvement of therapeutic gene delivery systems with a strong emphasis on development of regulated and targeted vector systems and 3) translation of the preclinical studies into clinical protocols and trials. Cancer gene therapy has, indeed, proceeded to world wide clinical trials and over half of these trials are aimed against five forms of cancers: melanoma, leukemia, prostate-, ovary- and squamous cell carcinoma of the head and neck (Gottesman, 2003).

In cancer gene therapy, tumor growth can be inhibited using different approaches (see summary in **table 3**). Tumor suppression can be achieved by inhibiting the hyperactive oncogenes or by restoring the insufficiently working tumor suppressor genes. The use of tumor suppressor genes and oncogenes in cancer gene therapy can be problematic, because they are not the only contributors to the malignant phenotype. In fact, no single gene has been identified that is defective in all human cancers. However, promising results with tumor suppressor gene p53 have been published in the treatment of non-small cell lung cancer and squamous cell carcinoma of head and neck (Clayman et al., 1999; Swisher et al., 2003). The efficacy of p53 is enhanced by its ability to induce anti-angiogenic features by down-regulating vascular endothelial growth factor (VEGF) (Nishizaki et al., 1999). Inactivation of hyperactive oncogenes has been successfully achieved with the current methodology (McCormick, 2001). One of the latest methods used for down-regulating the function of genes is RNA interference with synthetic siRNAs (short interfering RNA). This method has been shown to be effective in blocking the oncogene expression in tumor cells (Tuschl and Borkhardt, 2002).

Approaches independent of the genetic background of a malignant cell may in many cases be more useful and therefore these anti-angiogenetic-, immuno-, chemoprotective-, viro- and suicide gene -therapies have become more popular. Anti-angiogenetic therapies take advantage of the vascularization that is essential for tumor growth. The formation of blood vessels in tumors can be suppressed by inhibiting the expression of angiogenic proteins or introducing the anti-angiogenic proteins into cancer cells (Wannenes et al., 2005). One immunotherapy approach is to target the host immune system against malignant cells by inducing expression of tumor associated antigens in immunomodulatory cells. Another approach is to use cytokines to achieve boosted immune response against the cancerous cells (Ochsenbein, 2002). Chemoprotective therapies differ from the other cancer gene therapy forms in the way that healthy tissue is treated to make it more resistant against high doses of chemotherapy. An earlier finding of virus infection's ability to inhibit tumor formation (Huebner et al., 1956) has been exploited in recent cancer gene therapy studies. This so called virotherapy takes advantage of virus-mediated oncolysis, where replication of a mutant virus destroys the infected tumor tissue. These viruses can discriminate tumor tissue from normal tissue i.e. when they reach the normal tissue surrounding the tumor, then their spreading is aborted (Alemany et al., 2000; Kirn et al., 2001). For example, with adenoviruses, this tumor-selective action is based on mutations in E1A or E1B genes that limit the virus replication to cells that are defective in their p53 or retinoblastoma (Rb) pathways. Since these pathways are dysfunctional in many different tumor types, oncolytic adenovirus mutants are potential agents against a wide variety of malignancies.

Table 3. Different strategies for cancer gene therapy

Gene therapy strategy	Example gene	Reference
Tumor suppressor gene (compensation for defective expression by augmentation of a functional gene)	p53	(Kuball et al., 2002; Roth et al., 1998; Schuler et al., 2001; Schuler et al., 1998; Swisher et al., 2003)
	BRCA1	(Holt et al., 1996; Tait et al., 1999; Tait et al., 1997)
	RB	(Nikitin et al., 1999; Riley et al., 1996)
Oncogene (inhibition of overexpressed genes by different means)	ERBB2	(Alvarez et al., 2000; Czubayko et al., 1997; Lui et al., 2001)
	KRAS	(Alemany et al., 1996; Kazuteru Hatanaka, 2004; Miura et al., 2005)
Anti-angiogenesis (inhibition of tumor vasculature)	VEGF	(Im et al., 2001; Kong et al., 1998)
Immunotherapy (immune-based destruction of tumor cells)	IL-2	(Iwadate et al., 2005; Iwadate et al., 2000; Stewart et al., 1997; Stewart et al., 1999; Trudel et al., 2003)
Chemo-protective therapy (protection of bone marrow cells from high doses of chemotherapy)	MDR1	(Abonour et al., 2000; Cowan et al., 1999; Eckert et al., 2000)
Virotherapy, oncolysis (destruction of tumor cells by virus replication)	adenoviruses herpesviruses	(Kirn, 2001; Reid et al., 2002) (Markert et al., 2000; Shah et al., 2003)
Suicide gene therapy (destruction of tumor cells by expression of a prodrug-activating gene)	HSV-TK	(Pulkkanen and Ylä-Herttuala, 2005; Ram et al., 1997; Sandmair et al., 2000)
	CD	(Kuriyama et al., 1999a; Zhang et al., 2003)

BRCA; breast cancer, RB; retinoblastoma, ERBB; v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 VEGF; vascular endothelial growth factor, KRAS; Kirsten rat sarcoma viral oncogene homolog, MDR; multiple drug resistance, CD; cytosine deaminase, HSV-TK; herpes simpex virus thymidine kinase.

2.2.1 SUICIDE GENE THERAPY

Cancer arises from a multistep process involving a variety of genetic abnormalities. In order to treat all errors in the genetic code, replacement or correction of several genes would be required.

Hence, approaches independent of the target cell genome could be more effective at eliminating transformed cancer cells. Suicide genes have been studied as an elegant approach for cancer gene therapy. The aim of this approach is to create artificial differences between the normal and malignant cells in their sensitivity to certain prodrugs (Pope et al., 1997). The enzymes encoded by suicide genes can convert prodrugs with low inherent toxicity into a toxic compound. An additional advantage of this type of therapy is that the toxic form of prodrug can often diffuse into the neighboring cells. This so called bystander effect reduces the proportion of tumor cells that need to be transduced for tumor eradication. There are nowadays over ten different prodrug activating approaches available, utilizing enzymes derived from bacteria, yeast or viruses. All these approaches work through disruption of DNA synthesis, a process which is particularly active in all cancer cells (Aghi et al., 2000). The concept of suicide gene therapy is shown in **Figure 2**.

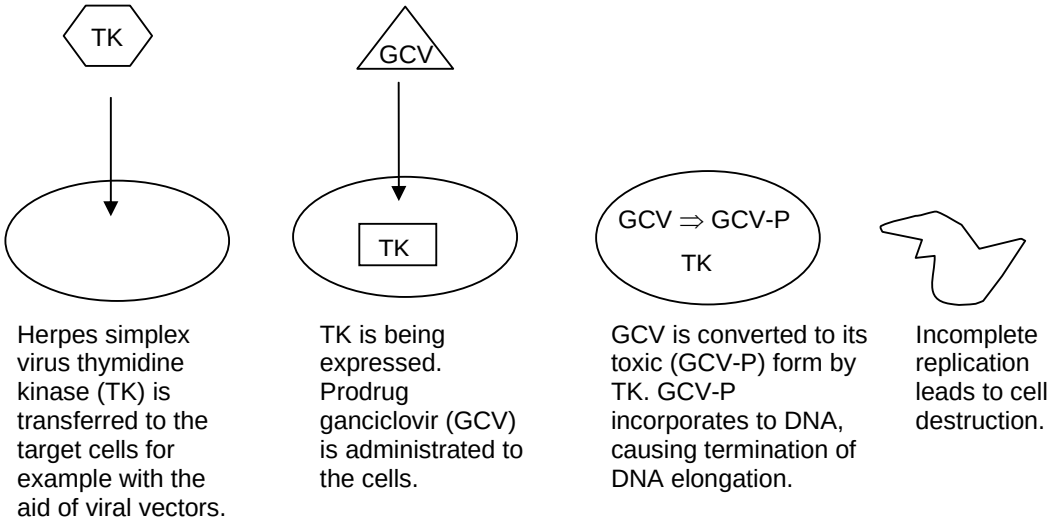
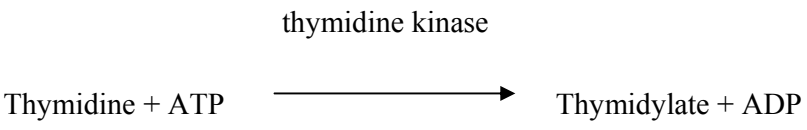


Figure 2. Schematic illustration of suicide gene therapy.

Viral vectors are often used to deliver suicide genes into cancer cells. After delivery, the suicide gene should be expressed at a relatively high level to provide antitumor activity. The vectors are, in most cases, delivered directly into the tumors or alternatively into its surrounding tissue, whereas the prodrug can be administered systemically. The most widely studied suicide gene therapy form is the herpes simplex virus thymidine kinase/ganciclovir suicide gene therapy approach (Moolten, 1986).

2.2.1.1 HERPES SIMPLEX VIRUS THYMIDINE KINASE/GANCICLOVIR GENE THERAPY

Cellular thymidine kinase (EC 2.7.1.21) is a key enzyme in the pyrimidine salvage pathway catalyzing the transfer of γ-phosphate from ATP to thymidine to produce thymidylate (TMP).



Herpes simplex virus thymidine kinase (HSV-TK) differs from its eukaryotic counterparts by its ability to phosphorylate a broad range of guanosine analogues, such as ganciclovir (GCV), acyclovir (ACV), buciclovir and penciclovir (Chen et al., 1979; DeClercq, 1984; Field et al., 1983; Miller and Miller, 1980). In the late 70's, several research groups independently discovered that these nucleoside analogs inhibited the replication of herpes virus in infected cells with low host cell toxicity (Fyfe et al., 1978). Toxic derivatives of nucleoside analogues were not found in cells infected with thymidine kinase-deficient herpes simplex virus strain (Cheng et al., 1983b; Elion et al., 1977; Smith et al., 1982) and it was therefore concluded that the toxic effect of analogues resulted from the activity of viral thymidine kinase.

A few years after the discovery of the connection between viral thymidine kinase and nucleoside analogues, Moolten and coworkers (1986) decided to test herpes simplex virus type 1 thymidine kinase as a cancer controller. The idea was to create tissue mosaicism for drug sensitivity and thereby make the tumor cell population different from the normal cell population. In their study, HSV-TK was transferred to murine sarcoma cells by calcium phosphate precipitation, after which the cells were inoculated into mice. The results were promising because a complete regression of the tumors in mouse was achieved after GCV treatment. To improve this idea, Moolten and Wells (1990) showed that this approach could be used *in vitro* and *in vivo* with retroviral vector mediated transduction of HSV-TK gene. This treatment was also tested by Culver et al. (1992) who demonstrated efficient brain tumor regression with rats carrying intracranial tumors. In order to achieve tumor regression, retrovirus vector producing cells were injected into the tumors, followed by intraperitoneal administration of ganciclovir. Since then, HSV-TK has become one of the most extensively studied suicide genes in cancer gene therapy research.

It is known that once the prodrug is phosphorylated by the HSV-TK, cellular kinases phosphorylate it into a triphosphate form that inhibits DNA synthesis (Kokoris and Black, 2002). The ability of GCV to block DNA chain elongation is based on the lack of the 3' -hydroxyl group that is required for formation of the phosphodiester bonds in the chain-elongation reaction. Consequently, the use of GCV in thymidine kinase positive cancer cells results in the appearance of small, non-functional fragments of DNA (Cheng et al., 1983a; McGuirt and Furman, 1982). DNA fragmentation caused by GCV triphosphate incorporation may also be responsible for the initiation of other mechanisms related to tumor eradication, such as the induction of apoptosis (Freeman et al., 1993).

When induction of apoptosis was studied more thoroughly, HSV-TK/GCV appeared to induce the accumulation of p53 and increase cell surface expression of the death receptors (Beltinger et al., 1999). Furthermore, the death receptor pathway was found to induce caspase activity, which leads to mitochondrial perturbations including the loss of the mitochondrial membrane potential and the release of an apoptogenic protein, cytochrome c, from mitochondria. Mitochondrial function during apoptosis is controlled by Bcl-2 family proteins and overexpression of Bcl-2 has been shown to inhibit HSV-TK/GCV induced mitochondrial perturbation (Kroemer, 1997). Thus, this system may not be very efficient in tumors that overexpress Bcl-2 (Beltinger et al., 2000). When using the HSV-TK/GCV treatment regimen, also other types of mitochondrial damage have been reported. For example, the triphosphate form of GCV can be incorporated into mDNA, causing mitochondrial dysfunction in nondividing cells (Herraiz et al., 2003).

In addition to apoptosis, there is evidence that immunological mechanisms are involved in tumor eradication induced with the HSV-TK/GCV system. Immunohistological analysis of 9L brain tumors showed that after successful HSV-TK/GCV treatment, there was a predominance of macrophages/microglia and CD8+ T-cells in the tissue surrounding the tumors. In the same study, GCV treated rats rejected repeated injection of syngeneic tumor forming cells, indicative of a strong anti-tumor immune response (Barba et al., 1994). In an animal study where a fusion of hygromycin phosphotransferase-thymidine kinase gene was used to transduce tumor forming 9L cells, a significant tumor regression was observed even without the presence of GCV (Tapscott et al., 1994). Furthermore, Kuriyama et al. (1999b) have reported that the HSV-TK system in fact needs a T-cell-mediated immune response to induce an efficient antitumor effect. They tested the HSV-TK cancer gene therapy approach in both immunocompetent and immunocompromized mice. A complete inhibition of tumor formation was achieved in the immunocompetent animals, whereas no significant tumor suppression was observed in the

athymic mice. Failure in tumor regression in nude mouse has also been described by others (Zhang et al., 1997). On the other hand, the lack of T-cell mediated immunity may not be the only reason for inefficient therapy in immunocompromized animal models, since Määttä and coworkers (2004) have shown a significant treatment response in nude mice exposed to HSV-TK/GCV gene therapy.

There are also a few limitations concerning the usage of the HSV-TK/GCV suicide gene therapy strategy. Konson and coworkers (2004) recently showed enhanced growth of tumors transduced with HSV-TK. They explained this phenomenon by the enhanced expression of cyclooxygenase-2 (COX-2) which leads also to the production of prostaglandin E_2 (PGE_2). Enhanced COX-2 expression has been shown to increase tumor growth (Fujita et al., 1998), invasiveness (Ohno et al., 2001) and resistance to chemotherapy (Taketo, 1998). Moreover COX-2 inhibitors have shown some efficacy at inhibiting tumor growth both *in vitro* and *in vivo* (Okajima et al., 1998; Reddy et al., 2000). GCV uptake and its low affinity to HSV-TK may also limit the clinical efficacy of this treatment form. Haberkorn et al. (1998) have concluded that GCV might not be the best substrate for HSV-TK due to its inadequate transport into the cells as well as the low levels of GCV phosphorylation. They showed that GCV uptake increased along with the percentage of HSV-TK expressing cells, which was considered to be a limiting factor in the *in vivo* situations, where HSV-TK expression may be low. They also pointed out that enhancing the affinity of HSV-TK to GCV would improve its therapeutic potential. Several reports about HSV-TK mutants with higher affinity for GCV than the wild type thymidine kinase have, indeed been published (Black et al., 1996; Drake et al., 1999; Hinds et al., 2000; Kokoris and Black, 2002; Mercer et al., 2002). It has also been noticed that sensitivity to GCV varies between different tumor cells lines (Beck et al., 1995; Ketola et al., 2004; Loimas et al., 2000b; Määttä et al., 2004). That can, at least partly, be explained by differences in the bystander effect between the cell types (Ishiimorita et al., 1997; Samejima and Meruelo, 1995).

2.2.1.2 BYSTANDER EFFECT

It was originally thought that for complete tumor eradication, each tumor cell had to express the suicide gene. With our current knowledge of the gene delivery methods, it is now appreciated that it is unrealistic to assume that every cell in the tumor can be transduced. In the first HSV-TK treatment with cultured cells, Moolten (1986) observed the phenomenon that also the HSV-TK negative cells were eradicated after GCV treatment. At that time, the phenomenon was not considered very important, but it has later turned out to be extremely important. Culver and

coworkers (1992) were the first to notice that even when there was only 10% of TK positive cells in the tumor mass, tumor growth was prevented in the presence of GCV.

Instead of an unknown type of 'vehicles', released from GCV treated, HSV-TK positive cells (Freeman et al., 1993), the transmission of bystander effect appeared to be due to delivery of phosphorylated forms of GCV from HSV-TK positive cells to wild-type cells (Ishiimorita et al., 1997). Experimentation with membrane bottomed chambers showed that the phosphorylated forms of GCV were not transmitted as soluble factors, instead cell-to-cell contact was needed to achieve efficient bystander (Samejima and Meruelo, 1995). It was anticipated that the bystander effect was mediated by gap junctions and, indeed, direct evidence of the relationship between gap junctions and bystander effect was obtained by Touraine et al. (1998a), who investigated calcein transfer between the cells. Calcein is known to be transferred through gap junctions and it can easily be detected via its fluorescence. In this study, cell lines with poor bystander effect did not show any evidence of intercellular transfer of calcein, indicating the lack of gap junctions. Recently, Gentry and co-workers (2005) observed with the bystander effect negative cell line HeLa that the transfer of GCV-TP may occur without any signs of a bystander effect. The absence of the bystander effect was not attributable to the lack of gap junction intercellular communication, but rather to the accelerated half-life of GCV-TP in bystander cells.

Cell to cell transfer of toxic metabolites of GCV is mostly facilitated through gap junctions (Mesnil and Yamasaki, 2000),but the possibility that other routes can supply bystander effects has also been suggested. For example, Princen and co-workers (1999) showed in rat colon adenocarcinoma that bystander mediated death was not inhibited by separation of TK positive and TK negative cells with a filter membrane. In another study, where the cells were exposed to forscolin, which enhances or stimulates gap junctions via an increase in the level of cAMP, inhibition instead of an increase, in the bystander effect was observed, suggesting that this represented gap junction independent bystander killing (Samejima and Meruelo, 1995). One of the earliest findings of gap junction-independent transfer of phosphorylated product of GCV was observed in human colon cancer cell line SW620. These cells had minimal gap junction dye transfer and low connexin expression, but they were highly sensitive to bystander killing (Boucher et al., 1998). The mechanism by which the bystander effect occurs in these cell line was characterized by Drake and co-workers (2000). SW620 cells metabolize GCV very efficiently and when these cells were mixed with bystander resistant cells, a dramatic increase in bystander mediated killing was observed. They proposed that high thymidine kinase expression is needed for efficient efflux of phosphorylated GCV from thymidine kinase expressing cells.

Gap junctions have been shown to be responsible for the bystander effect also *in vivo*. When tumors expressed exogenous connexin protein, bystander mediated tumor retardation was increased (Duflot-Dancer et al., 1998; Vrionis et al., 1997). Also, a number of chemicals like forscolin, cAMP and lovastatin, have been demonstrated to increase the numbers of gap junctions *in vivo* and consequently to improve the bystander effect. (Park et al., 1997; Touraine et al., 1998b).

2.2.1.3 HSV-TK IN COMBINATION WITH OTHER TREATMENT FORMS

HSV-TK/GCV gene therapy is still far from a perfect approach for treating cancer. Several strategies have been tested to enhance the therapeutic response of suicide gene therapy. One alternative way to obtain significant treatment results is to combine traditional cancer treatment methods with gene therapy. For example, after surgical removal of glioma, the mean survival was significantly higher in a group that received HSV-TK adenovirus into the tumor cavity (Sandmair et al., 2000). One of the earliest observations concerning the enhancing effect of suicide gene therapy to traditional therapy was published by Kim and coworkers (1994) . They demonstrated enhanced sensitivity to radiation in HSV-TK positive cells after GCV compared to non-transduced cells. Enhanced therapeutic effect has also been observed by combining prodrug therapies. Rogulski et al. (1997a,b), combined two widely used suicide genes, cytosine deaminase from E. coli (CD) and HSV-TK. They demonstrated an increased sensitivity to GCV and enhanced radiosensitivity *in vitro* and *in vivo* with the double suicide fusion protein (CD-HSV-TK). Another combination of two suicide systems, HSV-TK/GCV and CYP2B1/CPA, was studied in 9L subcutaneous tumors in athymic mice by Aghi et al. (1999). Tumor regression was achieved with oncolytic herpes simplex virus vector carrying both HSV-TK and p450 2B1. Compared to other treatment groups, only the combination treatment significantly reduced the tumor volumes. Enhancement of HSV-TK/GCV therapy was achieved also with simultaneous adenoviral delivery of uracil phosphoribosyltransferase (UPRT) which sensitizes cells to 5-fluorouracil (5-FU). In a murine model, this combination was further enhanced by radiotherapy, resulting in 90-100% cell death (Desaknai et al., 2003).

In addition to the combined use of two suicide genes, HSV-TK in combination with other genes has demonstrated increased efficacy. Simultaneous delivery of adenoviral vectors carrying either HSV-TK or monocyte chemoattractant protein-1 (MCP-1) to subcutaneous tumors of human hepatocellular carcinoma in nude mouse yielded enhanced cytotoxic effect (Sakai et al., 2001). Activation and recruitment of macrophages was shown to be associated with this combination. Enhanced activity of this system was also demonstrated with a colon cancer model in

immunocompetent mice via activation of innate and acquired immune responses (Kagaya et al., 2005). Another immune system related gene that has been combined with HSV-TK is cytokine granulocyte macrophage colony-stimulating factor (GM-CSF). GM-CSF has been a candidate gene for cancer vaccination due to its ability to activate antitumor immunity (Hsieh et al., 1997; Kayaga et al., 1999). Lee and coworkers (2004) tested this combination in a murine colon cancer model and their results indicated a synergistic enhancement of treatment with GM-CSF and HSV-TK.

One possibility to enhance HSV-TK cytotoxicity is manipulation of the pathways involved in induction of apoptosis. Tumor necrosis factor alpha, a protein that is known to possess a number of antitumor activities, was used in combination with HSV-TK (Moriuchi et al., 1998).Tumors transduced with TNFα/HSV-TK vector resulted in prolonged animal survival after GCV exposure. TNF-α's ability to enhance HSV-TK/GCV gene therapy can partly be explained by the induction of the antiapoptotic protein, NF-κB, which has various functions including inhibition of the nuclear FADD apoptosis pathway (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996). By inhibiting apoptosis in HSV-TK expressing cells, TNFα can prolong the life span of prodrug activating cells and therefore enhance the bystander effect (Waxman and Schwartz, 2003). Combining an antiapoptotic factor with suicide gene therapy was first demonstrated by Schwartz et al. (2002) in their study with p35 and p450 with prolonged activation of CPA by p450 expressing cells, leading to an increase in the bystander -evoked cytotoxicity. More effective treatment was achieved with HSV-TK and inhibition of antiapoptotic NF-κB (Moriuchi et al., 2005). This study showed that coexpression of NF-κB inhibitor, IKBA, can be used as a potential booster of HSV-TK suicide gene therapy. Inhibition of antiapoptotic NF-κB with mutant inhibitor IKBA has earlier been shown to enhance anti-tumor therapy by increasing apoptosis (Wang et al., 1999).

In addition to the combinations of different genes and HSV-TK/GCV therapy, there are a number of other interesting treatment combinations. For example, synergistic enhancement of HSV-TK/GCV cytotoxicity by hydroxyurea has been demonstrated. Hydroxyurea is a cheap, licensed drug that has been used in the treatment of some forms of leukemia for more than 30 years. Hydroxyurea acts as an inhibitor of ribonucleotide reductase, resulting in a depletion of essential DNA precursors. Boucher et al. (2000) showed that by depleting thymidine kinase's endogenous competitor, dGTP, then the GCV cytotoxicity appeared to be increased.

The same idea was investigated with gemcitabine (dFdCyd), a dCyd analogue that is known to interfere with DNA synthesis in its triphosphate form. The utility of gemcitabine is also improved by the fact that it is clinically relevant and widely used to treat solid malignancies (Blackstock et al., 1999a; Blackstock et al., 1999b; Eisbruch et al., 2001). Results with this combination were promising, showing improvement of HSV-TK cytotoxicity both *in vitro* and *in vivo* (Boucher and Shewach, 2005). A synergistic enhancement was also observed in a study with a combination of HSV-TK and interferon α2a (IFNα2a) (Whartenby et al., 1999). The mechanism of action in this study was not clear, but it may be related to the ability of IFNα2a to disrupt DNA damage assessment and/or repair. In a mouse model, combination treatment against melanoma was attempted with HSV-TK and histone deacetylase inhibitor drug FR901228 (Yamamoto et al., 2003). Histone acetylation has been shown to be an important regulator of gene expression at the transcriptional level (Grunstein, 1997) and prevention of histone deacetylation by certain inhibitors has been shown to reactivate and amplify expression of virally transduced genes (Chen et al., 1997) (Dion et al., 1997). Therefore the combination of FR901228, a well-characterized inhibitor of histone deacetylation and HSV-TK may represent a potential advance in suicide gene therapy.

Since HSV-TK/GCV gene therapy needs S-phase cells to work properly, it is apparent that if there were an increased number of S-phase cells in the tumor then this mass would improve the efficacy. When adenovirus E1a protein, which induces cell proliferation, was tested in combination with HSV-TK/GCV gene therapy, an increased cytotoxic effect was observed in mouse and human cell lines (Parada et al., 2003).

2.3 POLYAMINES

Polyamines putrescine, spermidine and spermine (**Figure 3**.) are small, straight chain, aliphatic, water soluble, organic cations found in all living cells with importance in optimal cell growth and viability in mammals (Jänne et al., 2004) in bacteria (Tabor et al., 1980) and yeast (Tabor et al., 1982) was proved in the early 1980's.

$NH_2CH_2\ CH_2\ CH_2\ CH_2\ NH_2$ Putrescine

$NH_2\ CH_2\ CH_2\ CH_2\ CH_2\ NH_2\ CH_2\ CH_2\ CH_2\ NH_2$ Spermidine

$NH_2\ CH_2\ CH_2\ CH_2\ NH_2\ CH_2\ CH_2\ CH_2\ CH_2\ NH_2\ CH_2\ CH_2\ CH_2\ NH_2$ Spermine

Figure 3. Natural polyamines.

At physiological pH, the amino groups of polyamines are protonated and that allows polyamines to take part in many physiological functions in the cell. Since they can undertake electrostatic interactions, polyamines bind nucleic acids and nucleotide triphosphates and other acidic substrates in cells (Feuerstein et al., 1990; Igarashi and Kashiwagi, 2000) and they are implicated in protein translation, membrane stability, cell proliferation and differentiation (Tabor and Tabor, 1984).

In order to maintain the cellular balance in polyamine pools, the biochemical reactions involved in polyamine metabolism are under tight control, since a decrease in polyamine levels can interfere with cell growth and excess levels are toxic (Jänne et al., 2004; Thomas and Thomas, 2001). Regulation is performed via *de novo* synthesis, interconversion, terminal degradation, excretion and uptake of polyamines.

The precursor of other polyamines, putrescine (Put), is formed from amino acid L-ornithine in a decarboxylation reaction catalyzed by L-ornithine decarboxylase, (ODC; EC 4.1.1.17) the first enzyme in polyamine biosynthesis pathway. The other amino acid in the biosynthesis of polyamines is L-methionine, which is first converted to S-adenosyl-L-methionine (AdoMet) by methionine adenosyltransferase (MAT; EC 2.5.1.6). S-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.50) further decarboxylates S-adenosylmethionine to decarboxylated AdoMet (dcAdoMet). An aminopropyl group from dcAdoMet is then transferred to either putrescine or spermidine in reactions catalyzed by spermidine synthase (EC 2.5.1.16) or spermine synthase (EC 2.5.1.22), respectively (**Figure 4**.). ODC and AdoMetDC are rate limiting enzymes of this biosynthetic pathway, and therefore under many regulatory mechanisms, transcriptional, translational and posttranslational (Jänne et al., 1991). In addition to being formed by biosynthesis, polyamines are also obtained from food and other sources such as intestinal bacteria (Sarhan et al., 1989).

Despite the irreversible nature of the carboxylation reactions in the polyamine biosynthesis, the higher polyamines, spermidine and spermine, can be converted back to putrescine. The first step in polyamine catabolism is acetylation of spermidine or spermine to form N^1-acetylspermidine or N^1-acetylspermine. In this reaction, the acetyl group from acetyl-CoA is transferred to spermidine or spermine by an inducible enzyme, spermidine/spermine acetyltransferase (SSAT; EC 2.3.1.57). Acetylated polyamines are substrates to constitutively expressed polyamine oxidase (PAO; EC 1.5.3.11), which means that SSAT is the rate controlling enzyme in the back-conversion pathway. Interestingly, Niiranen et al. (2002) described a SSAT independent conversion of spermine to spermidine in SSAT deficient mouse embryonic stem cells. The

recent discovery of a novel enzyme, spermidine oxidase (SMO; EC 1.5.3-) was shown to be behind this phenomenon (Vujcic et al., 2002).

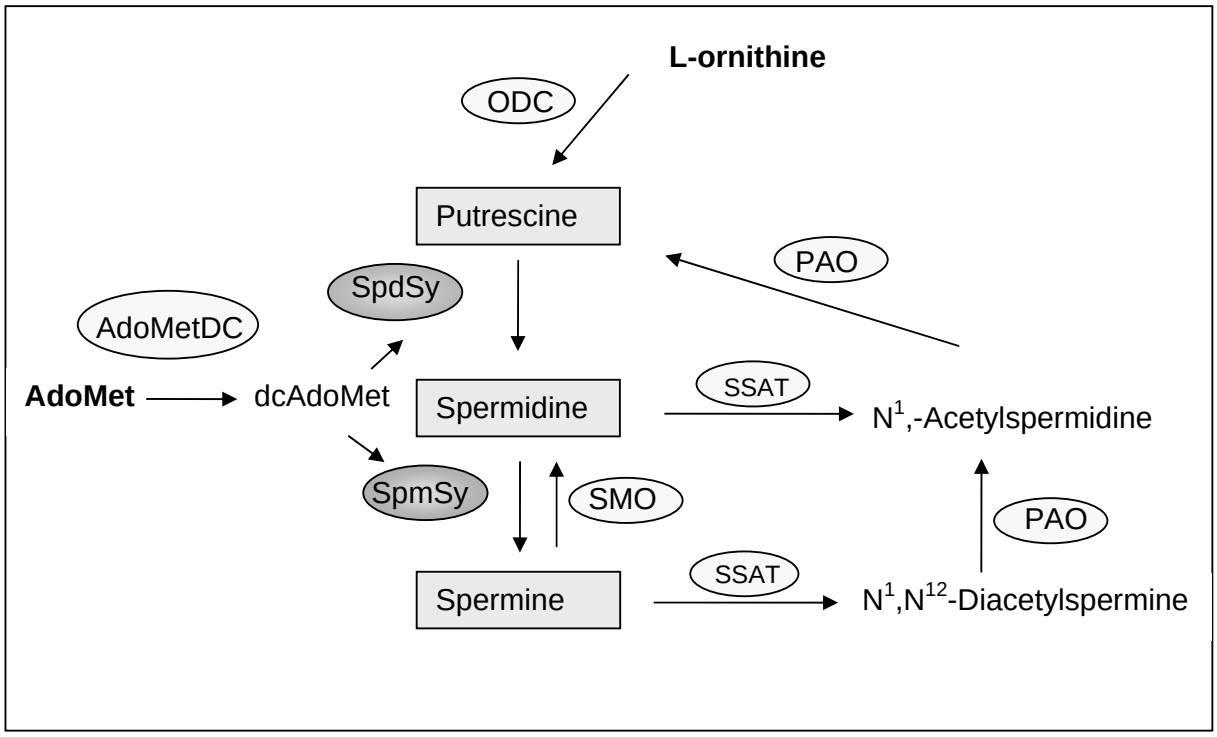


Figure 4. An overview of polyamine metabolism in mammals. Ornithine is produced from arginine (a product of the urea cycle) and further carboxylated to putrescine by ornithine decarboxylase (ODC). Spermidine synthase (SpdSy) converts putrescine to spermidine and spermine synthase (SpmSy) further to spermine. Polyamine oxidase (PAO) back-converts spermine and spermidine to spermidine and putrescine via acetylation by spermidine/spermine N^1-acetyltransferase (SSAT). Spermine oxidase (SMO) is capable of directly converting spermine to spermidine.

Cells need polyamines for active cell cycling. Similarly to the cyclins, polyamines are also detected in a cyclic manner during the cell cycle. The rate limiting enzyme in polyamine biosynthesis, ODC, correlates strongly to cell proliferation activity. The maximums of ODC activity are detected at G1/S transition and in S/G2 transition and as a consequence of ODC inhibition, the cell cycle is blocked in transition of G1 and S or into S-phase (Fredlund and Oredsson, 1996; Oredsson, 2003).

2.3.1 UTILITY OF POLYAMINES IN CANCER THERAPY

Elevation of polyamine concentrations and activation of their biosynthetic enzymes are essential and not only a consequence of cell proliferation in rapidly growing and neoplastic tissues. Polyamine homeostasis is well-orchestrated and controlled at the cellular and organ level and

disturbance of this delicate balance causes problems, often contributing to the development of malignancies. One of the earliest reports of polyamines associated to cancer originated from the late 1960's when Russell and Snyder (1968) reported high levels of ODC in human STAT-1 sarcoma. At that time, it was anticipated that information concerning polyamine biosynthesis and control pathways would have enormous industrial and medical/clinical applications. A large number of polyamine biosynthesis inhibitors, catabolism stimulators and polyamine analogs have been tested during the past 25 years in the search for a potent treatment for cancer (Wallace et al., 2004). The usefulness of polyamines in clinical applications has, however, faced many problems, apparently due to the complexity of the polyamine regulation systems. Therefore, no routine clinical applications have emerged so far.

Direct evidence of the relationship between polyamines and cancer control genes has been described in familiar adenomatous polyposis (FAP). In this form of hereditary predisposition to colon cancer, a tumor suppressor gene APC (adenomatous polyposis coli), is mutated leading to overexpression of oncogene c-Myc and one of its target genes, ODC (Luk and Baylin, 1984). ODC inhibition and gene therapy were combined in a study with human prostate cancer cells *in vitro* and *in vivo*. The antitumor effect of ODC inhibition was demonstrated after adenoviral delivery of antisense ODC (Zhang et al., 2005).

2.3.1.1 SELECTIVE ENZYME INHIBITORS AS CANCER CONTROLLERS

Polyamine biosynthesis has no alternative pathway in mammalians and there are very low levels of circulating polyamines available for tumors. Therefore it was thought that inhibition of polyamine biosynthesis could be a meaningful target for cancer treatment. The first tested targets were ODC and AdoMetDC. There are two known drugs targeted to those enzymes; α-difluoromethylornithine (DFMO) (Metcalf, 1978) and methylglyoxal bis(guanylhydrazone) MGBG (French et al., 1960).

DFMO inhibits irreversibly ODC, by binding to the active site at lysine 69 and cysteine 360 (Poulin et al., 1992). Cultured cells, treated with DFMO, showed clear depletion of putrescine and spermidine while the spermine pool remained unaffected, leading to growth arrest of the cells (Gerner and Mamont, 1986). DFMO interrupts the cell cycle in G1, but also prolonged S-phase has been indicated (Fredlund and Oredsson, 1996). A reduction in the rate of cell proliferation using DFMO does not usually lead to cell death when used at clinically relevant concentrations. DFMO's potency as an anticancer drug has been demonstrated in a variety of epithelial cancer animal models (Meyskens and Gerner, 1999). For example, mouse colon tumor

formation was shown to be inhibited by DFMO (Kingsnorth et al., 1983; Tempero et al., 1989). Recently, several publications have appeared indicating that DFMO can be used as a suppressor of invasion and metastasis in hormone-independent breast cancer (Manni et al., 2005; Richert et al., 2005). This effect of DFMO was linked to activation of the mitogen-activated protein kinase (MAPK) pathway indicated by increased ERK phosphorylation (Manni et al., 2004). Since ERK is linked to increased production of thrombospondin-1, an extracellular matrix protein with anti-invasive and antimetastatic properties, its inhibition could reverse DFMO's suppressive effect against invasion. All these features have been detected at the same time with administration of DFMO, but the actual mechanisms remain unknown. From a pharmacological point of view, DFMO is well tolerated even at high doses and long treatment times (O'Shaughnessy et al., 1999). One known side effect is temporary hearing loss, when the daily dose exeede 2 g/kg (Nie et al., 2005). However, there has been only one case report published describing this symptom (Lao et al., 2004).

MGBG inhibits another key enzyme, AdoMetDC, in polyamine biosynthesis (Corti et al., 1974; Williams-Ashman and Schenone, 1972). This drug can cause depletion in spermidine and spermine pools in parallel to the accumulation of putrescine (Porter et al., 1980). Although MGBG results in an inhibition of the cell growth, its utility in cancer therapy is limited by the toxic side effects, most notably the dramatic changes in mitochondrial structure and function (Knight et al., 1984; Pleshkewych et al., 1980).

Given the ubiquitous nature of polyamines in cellular function, these specific enzyme inhibitors have thus far shown only a moderate clinical utility. Nevertheless, they have revealed that by inhibiting key enzymes in the polyamine biosynthesis pathway, cell proliferation can be efficiently blocked. Their limited efficacy has led to the development of polyamine analogues that can compete or inhibit more than one enzyme involved in polyamine metabolism (Wallace and Fraser, 2003).

2.3.1.2 STRUCTURAL ANALOGUES

Similarly to high levels of natural polyamine concentrations, structural polyamine analogs are able to down-regulate the polyamine biosynthetic enzymes ODC and SAMDC or up-regulate SSAT and stimulate the export of polyamines (Porter et al., 1990). Early investigations with analogues revealed that the growth inhibition seen in non-small cell lung carcinoma and melanoma cell lines was due to decrease in polyamine which was attributable to the dramatic elevation in SSAT activity (Casero et al., 1989; Porter et al., 1991). In later studies, especially

N^1-N^{11}-diethylnorspermine (DENSPM) has been shown to increase the SSAT level at both transcriptional (Fogel-Petrovic et al., 1993) and translational levels (Parry et al., 1995). However, the response to analogues varies between cell types, causing cell specific effects. For example, in two lung cancer cell lines, there was a 1000 fold difference in N^1,N^{12}-bis(ethyl)spermine (BESPM) -induced SSAT activity.

3 AIMS OF THE STUDY

The general aim was to evaluate whether HSV-TK/GCV suicide gene therapy can be enhanced by manipulating the cell cycle phase distribution of tumor cells and tissues.

The specific aims were

1. To verify the hypothesis in cultured cells: can DFMO induce a reversible cell cycle block and can this phenomenon be used to enhance the HSV-TK/GCV suicide gene therapy approach? (**I**)

2. To establish an animal tumor model for *in vivo* evaluation of the combination. (**II**)

3. To test the combined treatment of DFMO and HSV-TK/GCV in the animal model. (**III**)

4. To test other polyamine homeostasis- and cell cycle altering drugs for further characterization of the enhancing effect. (**IV**)

4 MATERIALS AND METHODS

4.1 ANIMALS (II, III)

Female Fischer rats (**II**) or NMRI nu/nu mice (**II**, **III)** aged 8 weeks were purchased from Harlan, Netherlands. To anesthetize the animals before tumor implantation or viral transduction, fentanyl-fluanisone - midazolam was used. During the experiments animals were kept in the National Laboratory Animal Centre, University of Kuopio or in specific animal rooms at A.I.Virtanen Institute. All procedures concerning animal experiments were approved by the Animal Care and Use Committee of the University of Kuopio.

Tumor inoculation. To establish tumors from a cancer cell line, $6x10^7$ cells were suspended in 1 ml of OptiMEM (Gibco, BRL, Life Technologies, UK). Fifty µl (equal to $3x10^6$ cells) of suspension was inoculated subcutaneously using a 1 ml syringe equipped with 26G needle onto the back of each animal. One animal carried 2 or 4 tumors. When experiments involved ex vivo transduced cells, TK-GFP positive cells were inoculated into the right side and parental cells into the left side of the animal's back. After tumor inoculation, the growth rate of tumors was monitored by measuring the dimensions with a ruler. In the therapy experiments, animals were killed after GCV treatment and tumors were measured by weight.

Gene transfer. Ex vivo gene transfer was performed in cell culture conditions with lentiTK-GFP or lentiGFP. Prior to the inoculation, the number of positive cells was determined by flow cytometry. AdenoTK-GFP was used for intratumoral gene transfer and injected into tumors with a Hamilton syringe.

GCV treatment. Animals carrying either *ex vivo* or *in vivo* transduced tumors received ganciclovir for 7- 15 days, 30mg/kg/d (**II**) or 50mg/kg/d (**III**). GCV (Hoffmann-La-Roche AG, Grenzach-Wyhlen, Germany) treatment was started 4 days after virus transduction or in the case of *ex vivo* transduced cells, as soon as the tumors reached a diameter of 4 mm. In combination studies with DFMO (ILEX Oncology, Inc., San Antonio, TX), GCV treatment overlapped with DFMO by 2 or 5 days (**III**).

4.2 CELLS

Cell lines. All cell lines (**Table 4.**) were grown in DMEM (Dulbeccos's modified Eagle medium; Gibco) with high glucose and 10% fetal calf serum (Gibco), 2 mM glutamine (Gibco), 2 mM sodium pyruvate (Gibco) and 50µg/ml gentamicin at 37° C in the presence of 5% CO_2.

Table 4. The cell lines used in this thesis.

Cell line	reference
Rat gliosarcoma **9L** (ATCC CRL 2200)	I, II, III, IV
Human lung adenocarcinoma **A549** (ATCC CRL 185)	I
Human lung adenocarcinoma **NCI H23** (ATCC CRL 5800)	I
Human prostate carcinoma **DU-145** (ATCC HTB 81)	II
Human glioma **U-251 MG** (JCRB IFO50288)	IV
Human cervix cancer **Hela** (ATCC CCL-2)	II
Human embryonic kidney **293T**	II

Drug treatments. Drugs used in cell cycle inhibition experiments (I, IV) are listed in **table 5**. The used concentrations were experimentally determined to be non-toxic but still efficient at evoking changes in the cell cycle phase distribution.

Table 5. Drugs used to manipulate the cell cycle.

Drug	Used concentration	Properties
Aphidicolin	1 µg/ml	DNA polymerase inhibitor, prevents elongation of S-phase
DENSPM	50 µM	Activation of polyamine catabolism by inducing SSAT
DFMO	1 mM	Inhibitor of ODC
Hydroxyurea	0.5 mM	Ribonucleotide reductase inhibitor, chemotherapeutic agent, blocks cells in G1 phase
Lovastatin	60 µM	HMG-CoA reductase inhibitor, cholesterol lowering agent, also anti-cancer properties, arrests cells in G1 phase or causes growth retardation in cell lines
Mimosin	0.5 mM	Tyrosine analog, zinc chelator - inhibits zinc-inducible genes, arrests cells in G1 or early S phase
Resveratrol	50 µM	Phytoalexin found in plants, antifungal and anticancer properties, arrests cells in G1- or S phase

4.3 VIRAL VECTORS

Production of used viruses was carried out as described Loimas et al. (Loimas et al., 2000a). Briefly, lentiviral vectors were produced in 293T cells by cotransfection of three plasmids (vector, helper and envelope). Adenoviral vectors were propagated in 293 cells. Titers of all vectors were determined with flow cytometry. The viral vectors used are listed in **table 6**.

Table 6. The viral vector used in this thesis.

Viral vector	Properties	Ref.
Lenti TK-GFP	VSV-G pseudotyped, TK-GFP fusion gene, CMV promoter	I,II,III, IV
Lenti GFP	VSV-G pseudotyped, GFP gene, CMV promoter	I
Adeno TK-GFP	ΔE1/E3, serotype 5, TK-GFP fusion gene, CMV promoter	I
Adeno GFP	ΔE1/E3, serotype 5, GFP gene, CMV promoter	I

4.4 ANALYTICAL METHODS

Histology. For histological analyses, half of the tumor was cut off and paraformaldehyde fixed. Four µm sections were stained with Hematoxylin-eosin (II). Proliferating cell nuclear antigen (PCNA) immuno-histochemistry (III) was done to determine the cell proliferation activity in the tumor tissue.

Flow cytometry. For flow cytometric analyses of tumor samples, the tissues were first minced with a scalpel and then digested with an enzyme mixture containing 0.002% DNase I, 0.1% collagenase and 0.01% hyaluronase in OptiMEM for two hours. Samples were washed three times with PBS and then pipetted through a filter (35µm mesh) into Falcon cell-strainer tubes to obtain single-cell suspensions, which were analyzed for transduction efficacy (GFP label, green fluorescence) or cell cycle phase distribution (PI label, red fluorescence).

Polyamines, ODC and SSAT activity. For polyamine and western blot analyses, each tumor was divided into two equal parts. Polyamine concentrations from tumor tissue were analyzed by an HPLC method (Hyvönen et al., 1992). The method was used to determine polyamines from cultured cells. ODC enzyme activity was assayed by the method of Jänne and Williams-Ashman (1971). A previously described method was used to determine SSAT activity (Libby, 1978)

Western blot analyses. PCNA was analyzed from tumor tissue by western blot. Ten µg of protein was separated in 12% SDS polyacrylamide gel. After transfer onto Invirolon™ PVDF membrane

(Invitrogen, Paisley, UK), the membrane was blocked with 5% milk powder in TBS (10mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with mouse monoclonal anti-PCNA (1:5000) coupled with HRP (horse radish peroxidase) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) . ECL plus Western Blotting Detection system (Amersham Bioscience, Buckinghamshire, UK) was used for detection. The Storm® 840 Imaging System was used to quantitate the amount of PCNA protein.

Cell cycle analyses. Propidium iodide staining was performed to analyze the effects of the drugs on the cell cycle. Cells exposed to the drugs were trypsinized and resuspended in culture medium. After centrifugation at 500 x g for 10 min, the cells were resuspended into ice cold GM buffer (6.1 mM glucose, 137 mM NaCl, 5.4 mM KCl, 1.5 mM $Na_2HPO_47H_2O$, 0.9 mM KH_2PO_4 and 0.5 mM EDTA), after which 3 ml of ice cold ethanol was gently added to each tube. After at least 12 h incubation, the cells were washed once with PBS containing 5 mM EDTA. Pelleted cells were stained for 1 hour at room temperature by adding 1 ml of PBS containing 30 µg/ml PI and 0.3 mg/ml RNase A. Prior to cytometric analysis, cells were filtered through mesh cap tubes. This method has been described by Prather et al (1999).

Ganciclovir sensitivity assay. To determine the enhancing effect of the drugs to HSV-TK/GCV gene therapy, a ganciclovir sensitivity assay was performed. Cells were transduced with lentiTK-GFP and the proportion of TK-GFP positive cell was analyzed by flow cytometry. The transduced cells were mixed with parental cells to obtain populations with varying proportions (0-30%) of TK-GFP positive cells (I) or a population with 15% of positive cells was used (IV). The cells were plated to 96-well plates (I) or 48 well plates (IV). Cells treated with different drugs were exposed for five days to different concentrations of GCV (0-100 µg/ml, I) or 1µg/ml of GCV (IV). After GCV exposure, the MTT assay was carried out to determine cell viability based on the mitochondrial activity (Cell Proliferation Kit I, Roche Diagnostics, Indianapolis, IN).

5 RESULTS

5.1 HSV-TK/GCV IN COMBINATION WITH DFMO OR SERUM DEPRIVATION - THE IN VITRO STUDY (I)

DFMO's effect on 9L cells

Different concentrations and incubation periods of DFMO were tested for their ability to interfere with cell growth, polyamine levels and cell cycle phase in 9L and 9L TK-GFP cell lines (**I**, Fig. 2 and Table I). One day incubation with 1mM DFMO was enough to cause growth arrest and collapse of putrescine levels. With the two day incubation, a clear absence of spermidine was detected but the spermine content remained at the control level and an additional third incubation day did not bring any additional benefits (**I**, Fig 2). Despite the evident growth arrest and effect on the polyamine content with DFMO, only a rather mild effect was seen in cell cycle analyses immediately after the different incubation times and concentrations (**I**, Table I). The results revealed also that no additional benefit was achieved when DFMO concentrations higher than 1mM were used. Therefore, further experiments were carried out with a two day incubation in the presence of 1 mM DFMO.

Subsequently, we analyzed events after DFMO removal. Proliferation of the treated cells was arrested until five days post-DFMO, after which they started to divide rapidly. The polyamine contents after DFMO withdrawal were also studied and restoration of spermidine levels was seen on day three but putrescine levels stayed low over the observation period of three days (**I**, Fig 3). Although no clear changes in the cell cycle phase distribution were detected during the incubation with DFMO, a small increase in S-phased 9L cells was seen at days two and three after removal of DFMO (**I**, Table II). This effect was not detected in the other tested cell line, DU-145 (**I**, Table V).

Synergistic cytotoxicity of DFMO and HSV-TK/GCV

It seemed likely that simultaneous exposure of 9L cells to DFMO and GCV would not lead to enhanced cytotoxicity, since DFMO caused arrest of cell division and recovery was initiated a few days later. In an attempt to achieve enhancement with DFMO, four different schedules for GCV treatment after DFMO exposure were tested (**I**, Fig 4.). In the first experiment setting, where GCV treatment was started 24h after removal of DFMO, the cells were actually protected from the cytotoxic effect of GCV (**I**, Fig 1.). An enhancement of HSV-TK mediated cytotoxicity was detectable, when DFMO was removed earlier (2, 3 or 4 days before GCV), the best

combination being: GCV treatment started four days after DFMO treatment. These experiments were carried out with two cell lines: 9L rat glioma and DU-145 human prostate carcinoma (**I**, Fig. 4 and 7). The enhancement appeared to be more prominent in 9L cells.

Effect of serum deprivation on the cell cycle and suicide gene therapy

Serum starvation experiments were done to test whether cell cycle arrest without any disturbance of the polyamine homeostasis could cause the same effect as DFMO. Serum depletion induced changes in the cell cycle phase distribution when the cells were grown for four days in 0.1% serum -containing medium compared to 10% serum in a standard culture medium (**I**, Table III). After two days in the reduced serum conditions, there were 79% cells in G0/G1-phase compared to 54% in that phase in the control group. Furthermore, the cell cycle phase distribution after release from serum starvation was different than that seen after DFMO treatment. Twenty four hours after restoring the normal growth conditions (10% serum), changes were seen in the proportions of G0/G1 and G2/M -phased cells compared to the control group (**I**, Table IV). Also, the proportion of S-phase cells was elevated from 18% to 33%. Despite the presence of these cell cycle effects, serum starvation turned out to be a weak enhancer of the HSV-TK/GCV cytotoxic effect. When 24h serum starvation was followed by GCV treatment, started on days 0, 1 and 2 after starvation, only marginal enhancement of cytotoxicity was observed at best (**I**, Fig 6.). The effect was most evident, when GCV treatment was started immediately after starvation.

5.2 SUICIDE GENE THERAPY WITH TK-GFP IN RODENT TUMOR MODELS (II)

To characterize viral vectors carrying the fusion construct of thymidine kinase and green fluorescent protein (TK-GFP) (Loimas et al., 1998) *in vivo,* two different tumor models were tested for their properties. TK-GFP or GFP alone was transduced either *ex vivo* or *in vivo* with the aid of a lentiviral or adenoviral vector. Two different animal strains were used in these experiments, Fischer 344 rats or NMRI nu/nu mice. Fischer 344 is an immunocompetent rat strain, from which the 9L cell line was isolated. Thus, 9L cells can form tumors in these rats. NMRI nu/nu mice are devoid of functional T-cell capabilities and many human cancer cell lines can form tumors in these animals.

Features of TK-GFP- and GFP- positive tumors in Fischer 344 rats

The TK-GFP fusion protein, expressed in *ex vivo* transduced subcutaneous tumors in Fischer rats, was capable of causing significant tumor growth retardation in the presence of GCV

(P = 0.006, n = 6). This result was obtained when administration of GCV was started immediately after tumor inoculation (**II**, Fig. 1). However, the results with tumors that were established almost two weeks before initiation of GCV were not as impressive i.e. treated TK-GFP tumors were not significantly smaller than the respective control tumors (**II**, Fig. 2). Due to the weak response in that experiment, TK-GFP expressing cells were monitored both in cell culture and in an animal tumor model. One salient feature in the immunocompetent rat model appeared to be the clearance of *ex vivo* transduced TK-GFP positive cells, even in the absence of ganciclovir. When the expression of TK-GFP was followed in cell culture conditions, no clearance was detected. Apparently, the poor response to the therapy that was started 11 days post tumor inoculation was due to this phenomenon i.e. the TK-GFP gene positive cells may have been partially eliminated from the tumors before initiation of GCV treatment. When GFP expressing cells were monitored *in vivo* or *in vitro,* no loss of transgene positive cells was detected (**II**, Fig. 3.), suggesting that the immune response was mounted against TK when T-cells are present.

To obtain a more efficient approach, we investigated gene transfer *in vivo* with an adenoviral vector carrying the TK-GFP fusion gene (**II**, Fig 6A and B). With a total amount of $3x10^7$ pfu AdTK-GFP, the resulting proportion of TK-GFP positive cells in 9L tumors was 25% (**II**, Fig. 6B). When GCV treatment was started four days post virus injection, a marked treatment result was obtained (**II**, Fig. 6A). In this experiment, it was also observed that the virus itself caused an antitumor response. When the expression of the TK-GFP transgene was followed, similar clearance of the TK-GFP positive cells was seen as with in *ex vivo* transduced tumors.

Features of TK-GFP in NMRI nu/nu mice

To confirm that the clearance of TK-GFP positive cells from tumors inoculated into Fischer rats was due to a functional immune response, we monitored the expression of the fusion protein in a partially immunodeficient animal model using human lung carcinoma cell lines A549 and NCI H23 (**II**, Fig. 5). No difference in clearance of TK-GFP positive cells *in vitro* vs. *in vivo* was seen. Although the proportion of TK-GFP positive A549 cells was initially reduced by 50%, the remaining level was maintained. The other cell line, NCI H23, maintained the initial proportion of the TK-GFP expressing cells, and again no difference was seen between *in vitro* and *in vivo* experiments.

5.3 DFMO - THE *IN VIVO* STUDY (III)

DFMO's effect on intratumoral polyamine levels and proliferation activity

Since a promising effect of HSV-TK/GCV with DFMO was seen in cell culture experiments (**I**), we sought to study it further in an appropriate animal model. NMRI nu/nu mice with subcutaneous tumors were chosen as the model due to the stability of the TK-GFP transgene expression (**II)**. First, the effect of DFMO on intratumoral polyamines and cancer cell proliferation was tested. The animals received 2% DFMO in their drinking water for one week. After DFMO administration, the tumors were analyzed at four time points for their ODC activity, polyamine content and PCNA activity (**III,** Fig.1, time indicates days post withdrawal of DFMO). The results showed a clear reduction of ODC activity. Putrescine and spermidine levels were reduced to one quarter of the control levels. When the tumors were analyzed after DFMO withdrawal, restoration of ODC activity and putrescine and spermidine levels was apparent in four days. Analysis of tumoral PCNA activity by Western blot technique revealed the same trend (**III**, Fig. 2.); the highest proliferation activity was detected four days after termination of DFMO treatment.

DFMO in combination with HSV-TK/GCV: effects on ex vivo transduced tumors

Two different schedules for combination treatment were tested to ensure that there would be a maximum number of simultaneously dividing cells at the initiation of GCV treatment (**III,** Fig 3A). The best treatment result was obtained when GCV treatment began 5 days after the DFMO regimen (i.e. an overlap of 2 days). Almost complete eradication of tumors was detected in that group. The result was significant ($P<0.05$) compared to the group that received only GCV (**III,** Fig. 3 B). Slight enhancement of HSV-TK/GCV cytotoxicity was also detected in the other combination treatment schemes where GCV was initiated two days later than DFMO.

5.4 ENHANCEMENT OF HSV-TK/GCV GENE THERAPY IN VITRO BY DIFFERENT MEANS OF CELL CYCLE MANIPULATION (IV)

Effect of N^1, N^{11}-diethylnorspermine (DENSPM)

To study the effect of polyamine depletion induced by other means than DFMO, the polyamine analog DENSPM was tested. DENSPM is an inducer of the polyamine catabolizing enzyme SSAT and incubation with DENSPM results in elevated SSAT levels and as a consequence, reduced polyamine pools. According to our results, DENSPM had only a slight effect on SSAT

activity in 9L cells, but in U-251 MG cells, SSAT activity was significantly elevated (**IV**, Table I). Consequently, changes in the polyamine contents were minor in 9L cells. In U-251 MG cells, however, the elevated SSAT activity caused almost complete polyamine depletion at 48 hours (**IV**, Table I). When the DENSPM effect on the cell cycle was investigated, there was no detectable change in either of the cell lines (**IV**, Fig. 2). Furthermore, when DENSPM was used in combination with GCV, no enhancing effect was detected in the 9L or U-251 MG cell lines (**IV**. Fig. 3).

Effect of aphidicolin, hydroxyurea, lovastatin, mimosin and resveratrol

Next, we tested other cell cycle altering drugs (aphidicolin, hydroxyurea, lovastatin, mimosin and resveratrol) and their effect on 9L and U-251 MG cells. All drugs except lovastatin were able to induce G0/G1-S-transition phase block. When the block was released ("0h" in **IV**, Fig 3.), aphidicolin-, hydroxyurea-, mimosine- and resveratrol -treated cells showed a rapid and transient increase in the number of cells in the S phase and a corresponding decrease of G0/G1 cells. In both cell lines, the proportion of S phase cells peaked in less than 8 hours after release from each drug. 9L cells attained equilibrium with regard to cell cycle distribution shortly after that time, whereas U-251 MG cells went through another aberrant cell cycle (**IV**, Fig. 3). Despite this effect on the cell cycle, none of the compounds seemed to enhance suicide gene therapy with HSV-TK/GCV. In all cases, the variation in cytotoxicity was attributed to the drug effect alone, not the synergy between the drug treatment and HSV-TK/GCV gene therapy (**IV**, Fig.4).

6 DISCUSSION

Despite a great deal of effort that has been expended to achieve clinically satisfactory results from cancer gene therapy and a number of clinical breakthroughs, there is still much work to be done before this technology will become an accepted part of clinical routine. The insufficient gene transfer efficiency is one of the most prominent obstacles in cancer gene therapy and many gene therapy researchers are nowadays focusing on developing new vector types (Kootstra and Verma, 2003). During past few years, increasing attention has been paid to conditionally replicative viruses (Alemany et al., 2000; Shah et al., 2003). Also vector targeting to cancer cells is undergoing intense development (Peng and Russell, 1999). The herpes simplex virus thymidine kinase-based suicide gene therapy approach has been thoroughly studied and its clinical efficacy is undisputable, but room for improvement remains (Pulkkanen and Ylä-Herttuala, 2005). Also, involvement of polyamines in the development of different cancer types has been demonstrated and inhibitors of various polyamine synthesizing enzymes have been tested in clinical trials (Gerner and Meyskens, 2004). Due to the theoretical synergy of suicide gene therapy and polyamine depletion, we wanted to test the interaction of these two approaches.

6.1 HSV-TK/GCV WITH DFMO; TESTS *IN VITRO*

HSV-TK/GCV suicide gene therapy needs active DNA replication in the target cells for proper function (Moolten, 1986). Theoretically, increasing DNA replication in the cells would lead to more effective therapy. One way to synchronize DNA replication in a cell population is to arrest the cell cycle to G1 phase and release the block. After release of the block, there should be temporarily more S phase cells in the population and as a consequence, more DNA synthesis. Various different chemicals have been shown affect the cell cycle, one of them being ODC inhibitor DFMO (Seidenfeld et al., 1981). Primarily DFMO causes polyamine depletion in cells, which also leads to growth retardation. That feature of DFMO has been utilized in cancer treatment experiments and clinical trials (Meyskens et al., 1998; Wallace and Fraser, 2004).

We tested whether DFMO could enhance the cytotoxic effect of the HSV-TK/ganciclovir gene therapy regimen. In the initial experiment where GCV treatment was started immediately after DFMO treatment, DFMO seemed to protect cells from the effects of GCV. That could be explained by a reduction in DNA synthesis caused by growth arrest induced by DFMO. Although the spermidine and spermine contents of the cells had reverted to normal level after 3 days, there were still insufficient polyamine levels for proper cell division, since the cells did not start to divide until 5 days after DFMO removal. The putrescine content in the cells was still very low at

3 days post-DFMO -induced growth arrest, indicating that most of the newly synthesized putrescine had been completely consumed for production of spermidine and spermine. We also found in the *in vivo* study that PCNA activity remained low after DFMO treatment until the putrescine levels were back in normal, indicating that cells require putrescine if they are to undergo proper cell division.

The cell cycle phase distribution was followed 3 days post DFMO treatment or serum deprivation. The results revealed accumulation of S phase cells on days 2 and 3 after DFMO removal, indicating that DFMO had caused prolongation of the S phase, since the cells did not start to divide until 5 days post DFMO. A more obvious cell cycle effect was caused in serum deprivation studies, where doubling in the numbers of S phase cells was observed 24h after returning serum back to the growth medium. More importantly, the duration of serum depletion -induced S phase cell accumulation was short and cells reached the normal cell cycle phase distribution after 48h of serum depletion. According to these cell cycle effects observed with DFMO and serum depletion, GCV treatment should be started several days post DFMO treatment or immediately after serum deprivation to achieve the full benefit from the increased pool of S phase cells.

Three different schemes were tested in attempts to find an optimal schedule for GCV during the increased DNA replication. In the case of DFMO, GCV treatment was started 2, 3 and 4 days post DFMO removal and in case of serum deprivation 0, 1 and 2 days post starvation. All combination treatment schedules with DFMO seemed to be more cytotoxic than GCV alone. The combination was the most efficient when GCV was started four days after DFMO removal. This fits well with the observation of initiation of cell proliferation activity 5 days after DFMO treatment. Serum deprivation was generally a weaker enhancer of TK/GCV suicide gene therapy. The best result with this combination was obtained when ganciclovir was added to the cells immediately after 24h serum deprivation.

These results supported the hypothesis that DFMO -induced polyamine depletion and the following cell cycle effects were responsible for the enhanced the cytotoxic effects of HSV-TK/GCV. We propose that the reason for DFMO being a better enhancer of suicide gene therapy than the serum deprivation was due to the extended period of the S phase that allowed longer time for incorporation of GCV-TP into replicating DNA. However, there might be other parallel effects caused by DFMO that we did not investigate. According to results of Whartenby et al., the accumulation of S phase cells caused by IFNα2a did not increase the GCV-TP incorporation to DNA, although an enhancement of HSV-TK/GCV cytotoxicity was achieved

(Whartenby et al., 1999). They speculated on the possibility that the cells were being retained in the S phase to repair the DNA damage caused by GCV incorporation and IFNα2a disrupted the damage assessment and/or repair, leading to enhancement of the effects of HSV-TK/GCV. Recent findings have indicated that DFMO -induced polyamine depletion can reduce apoptosis (Bhattacharya et al., 2005a; Bhattacharya et al., 2005b; Muscari et al., 2005).The combination of an antiapoptotic agent with suicide gene therapy of cancer may not initially seem rational. However, an enhancement of p450 suicide gene therapy was detected when the antiapoptotic factor p35 was co-introduced into tumor cells (Schwartz et al., 2002). Thus, the enhancing effect of DFMO to HSV-TK/GCV approach observed in our experiments could be a consequence of some antiapoptotic features of DFMO. For example, by providing an extended life span to HSV-TK positive cells, DFMO may enhance the bystander effect.

6.2 TK-GFP FUSION PROTEIN IN ANIMAL MODELS

Based on the work that has been previously done in our laboratory, i.e. the design of the TK-GFP fusion construct (Loimas et al., 1998) and its *in vitro* characterization in different cell lines (Loimas et al., 2000a; Loimas et al., 2000b), it was logical to expand the testing to *in vivo* properties of this fusion protein. Furthermore, this study was designed to verify which tumor model would be the most reliable and thus most suitable for in vivo testing of the DFMO in combination with HSV-TK/GCV. According to our results, TK-GFP worked as hypothesized and it allowed tracing of fluorescent cells from tumor samples with flow cytometry. The utility of GFP in histology was a challenge since expression levels in gene transfer experiments were too low and enhancement with labeled antibodies would have been necessary (data not shown). Also, detection of GFP from fresh tumor samples was complicated by the strong background fluorescence of tumor tissue. To avoid limitations of using a GFP as the marker, an imaging procedure with radiolabelled enzyme substrates for monitoring TK expression *in vivo* by positron emission tomography would have been one possibility (Tjuvajev et al., 1995). This would have allowed repeated non-invasive measurements from the same animal.

Ex vivo lentivirally transduced 9L tumors in an immunocompetent animal model

The properties of the thymidine kinase in the fusion protein were tested in GCV treatment experiments. The first experiments were performed with Fischer 344 rats inoculated with *ex vivo* transduced 9L glioma cells. Since we were focusing on the features and function of the fusion protein, the use of the *ex vivo* method minimized the variation in TK-GFP positive cell number between individual tumors. When tumors were inoculated with *ex vivo* transduced cells, it was

also easy to follow and compare the behavior of transgene expression *in vivo* and *in vitro* (**II**, Fig. 3 and 5). The initial tumor cell population in this study contained 55% of TK-GFP positive cells, but only a moderate treatment result was obtained when GCV treatment was started 11 days after tumor inoculation (**I**, Fig 2.). In an attempt to understand the reason for these poor results, we investigated whether there were still TK-GFP positive cells in tumors at 11 days from tumor inoculation. Our results demonstrated a clear reduction in the amount of TK-GFP positive cells in tumor tissue. When the expression of GFP alone was investigated, either in tumor tissue *in vivo* or in cell culture conditions, no clearance of positive cells was detected, suggesting that the TK domain of the fusion protein was responsible for the disappearance. In addition, when the cytotoxic effect of TK-GFP lentiviral vector was studied in 9L cell line, we found a low level transient apoptotic response, whereas with lentiviral vector harboring only GFP, no sign of apoptosis was seen. In conclusion, we observed some HSV thymidine kinase -related toxicity; in immunocompetent animals this could be the reason for the immune response evoked against TK-GFP positive cells. Accordingly, it was logical that better results were achieved when GCV treatment was started immediately after tumor transplantation (**I**, Fig.1 vs. Fig 2.).

In vivo transduction with adenoviral vector in immunocompetent animal model

To achieve a more effective cancer gene therapy approach, the efficacy of TK-GFP was also tested with *in vivo* transduced tumors using an adenoviral vector (AdTK-GFP). The transduction efficiency, with a total of $3x10^7$ pfu per injection was above 20%, making direct comparison of the treatment results to *ex vivo* transduced tumors difficult. Despite the lower proportion of positive cells, the treatment results were significant compared to the group that did not receive viral vector. Which may be attributable to a good bystander effect in the 9L cell line, as demonstrated earlier (Loimas et al., 2000a). Another reason could be toxicity or an immune response mounted against the vector, since tumor suppression was detected also in the group that received only vector. To test whether AdTK-GFP could induce apoptosis in cultured 9L cells, the consequences of transduction with two different multiplicity of infections (MOI 1 and 10) at three time points were monitored. At low MOI, some toxicity was observed during later time points, whereas with ten times higher MOI, viral toxicity was observed already at 24h post transduction. Thus, the vector effect may have provided additional efficacy in this tumor model.

Experiments with an immunodeficient animal model

Further studies were carried out in a nude mouse model to see if the clearance of TK-GFP positive cells was due to an immune response mediated by cytotoxic T-lymphocytes. No spontaneous elimination of TK-GFP was detected in *ex vivo* transduced tumors consisting of

NCI H23 (**II**) or 9L (**III**) cells, supporting the theory that the immune system plays a role in clearance. However, some questions arose from the data indicating eradication of TK-GFP positive cells in the A549 cell line. Positive cells disappeared both in cell culture and in tumors in nude mice. One explanation could be a strong interferon response in the A549 cell line in response to virus infection (Keskinen et al., 1999; Ronni et al., 1997). Thus, the positive cells may have been destroyed due to the influence of cellular first line defense rather than an adaptive immune responses. This is supported by GCV sensitivity experiments in another study yielding less impressive effect with the A549 cell line compared to other lung carcinoma types (Määttä et al., 2004).

Distant bystander

Due to the experimental design where one animal carried four tumors, two of them originating from parental cells and the other two from TK-GFP transduced cells, an interesting feature was detected. In the treatment group, also the non-transduced tumors became reduced in size. The difference between parental tumors in the control group versus treatment group tumors was clearly visible as shown in figure 2 (**II**). This phenomenon was clear when GCV administration was started 11 days after tumor inoculation. When the treatment was initiated against freshly inoculated tumors, this phenomenon was not seen. Studies *in vitro* have shown that the bystander effect is mediated through gap junctions (Ishiimorita et al., 1997; Touraine et al., 1998a), but our data indicates that there is some other mechanism operating behind this distant antitumor effect. This observation was also made by Bi and coworkers (1997). They showed that no distant bystander effect was seen in dexamethasone treated nude mice, pointing to the involvement of the immune response. According to their results, dexamethasone did not have any effect on tumor retardation of TK expressing tumors. A conflicting observation was recently published with dexamethasone in combination with HSV-TK/GCV gene therapy. These results pointed to a decreased bystander effect after reduction of the gap junction mediated intercellular communication in TK-expressing U87, C6 and LN18 cell lines after dexamethasone treatment (Robe et al., 2005).

Selection of the appropriate in vivo tumor model

Selection of the animal model depends on many factors. Different alternatives are available and understanding of pros and cons of each model is needed before one can make the right decision. The use of immunodeficient animals in gene transfer experiments is justified by the longer persistence and stronger expression level of the transgene (Quinones et al., 1996). Especially when new vector types and expression cassettes are being studied, this characteristic of the nude

animal is desirable. On the other hand, immunodeficient animals poorly represent the situation faced in clinical trials. Continued use of nude animal models may be due to requirements imposed by the local authorities. For example, the FDA (The United States of America Food and Drug Administration) requires inspection of experimental data from human cell lines and xenograft animal models before granting approval of a clinical trial protocol. In immunocompetent animal model, the immune system undoubtedly plays a role in the eradication of transgene expression and this phenomenon will influence the treatment results. There is also evidence that the role of the bystander effect, which is crucial in suicide gene therapy, is suppressed when nude mice models are used (Quinones et al., 1996).

6.3 HSV-TK/GCV WITH DFMO: TESTS *IN VIVO*

Since the results from in vitro studies with DFMO as an enhancer of HSV-TK/GCV suicide gene therapy approach were promising (**I**), the investigations were continued in an animal model. According to our *in vivo* studies (**II**), it was justified to choose the immunodeficient animal model to this proof-of-principle study with DFMO. Clearance of TK-GFP positive cells would have been inevitable after one week DFMO treatment, if *ex vivo* transduced tumors had been generated in an immunocompetent animal. Thus, if we had used animals with a normal immune system, we would need to have performed *in vivo* gene delivery, a technique which causes higher deviation of transgene expression between individual tumors and thus it would have been more difficult to obtain a reliable answer to our main question (i.e. can DFMO treatment significantly enhance HSV-TK/GCV antitumor effect?) .

The consequences of one week of treatment with 2% DFMO in drinking water on tumor polyamines, ODC activity and cell proliferation activity were determined at four time points after treatment. A clear decline of ODC activity and a decrease in the level of putrescine and spermidine were detected immediately after the treatment (**III,** Figure 1). A corresponding elevation in the values was detected on day 4 post DFMO removal. Western blot analysis with a PCNA antibody was carried out to determine the effect of DFMO on cell proliferation activity. The results were in line with the polyamine results, showing a decrease in proliferation activity immediately after DFMO treatment with the highest proliferation occurring 4 days later. In conclusion, DFMO was capable of inducing a reduction in ODC activity and consequent polyamine depletion in parallel with a decrease in the cellular proliferation activity. Since the restoration of cell proliferating activity took even longer than *in vitro,* we assumed that the synergy between DFMO treatment and HSV-TK/GCV could be achieved also *in vivo*.

To plan the GCV treatment schedule, we hypothesized that GCV should be already present in tumor cells at the time when the highest proliferation activity was detected. In this respect, we designed two different combination treatment models differing in the overlapping time of DFMO and GCV (**III** Fig. 3A). The overlapping times were chosen to be 2 and 5 days, on the basis of the results that indicated the highest proliferation activity should be observed four days post-DFMO. Furthermore, 2-5 days should be sufficient time for GCV to reach every cell in the tumor.

When tumors were analyzed after GCV treatment, a marked difference between the combination groups and the group that received only GCV was achieved. The best results were seen when the treatments overlapped by two days (**III** Figure 3). Careful tumor analysis after the combination regimen revealed that there were still 7% HSV-TK positive cells in GCV treated tumors whereas no HSV-TK positive cells were detected in the group that received only GCV. This observation supports the theory that DFMO has also antiapoptotic features that protected the HSV-TK positive cells in the tumors and aided in the bystander effect, leading to observed enhancement of HSV-TK/GCV suicide gene therapy.

6.4 FURTHER ANALYSES OF CELL CYCLE MANIPULATION IN COMBINATION WITH HSV-TK/GCV

In this study we wanted to determine whether activation of polyamine catabolism instead of inhibition of polyamine biosynthesis could enhance HSV-TK/GCV cytotoxicity. We also tested other drugs that had known effects on the cell cycle to verify if they could enhance the cytotoxic effect of HSV-TK/GCV. N^1,N^{11}-diethylnorspermine (DENSPM) is a symmetrically substituted analogue based on the structure of spermine. It was selected for our studies because of its known cytotoxic effect in tumor cell lines and its capability to induce the polyamine catabolic enzyme, SSAT and the consequent polyamine deprivation. Studies were carried out with two cell lines, 9L and U-251 MG. Induction of SSAT by DENSPM was tested first and it was discovered that DENSPM was unable to convincingly induce SSAT in the 9L cell line. The U-251 MG cell line responded in an expected manner with SSAT activity being elevated by 120 and 200 times at the 24h and 48h incubation timepoints, respectively. These results further confirm earlier findings showing that DENSPM has cell specific responses, particularly in its ability to induce SSAT activity (Casero et al., 1989; Casero et al., 1992). We continued the studies with DENSPM and examined its effects on the cell cycle distribution. The results suggested that there were no cell cycle effects in either cell line although the effects on SSAT activity and polyamine pools were altered in U-251 MG cells. This suggests that deprivation of polyamines is not sufficient to

induce any prominent cell cycle phase distribution changes. The effect of DENSPM on cell cycle distribution also appeared to be cell line specific since Alm et al. (2000) have shown in CHO cells that DENSPM causes accumulation of S phase cells.

Although no cell cycle effect with DENSPM was detected, it could not be ruled out that polyamine depletion itself in U-251 MG cell line was sufficient to enhance HSV-TK/GCV gene therapy. The enhancing effect of DENSPM on GCV treatment was investigated in both cell lines. Different schedules for initiation of GCV related to DENSPM treatment were tested similarly as in the study evaluating the synergy between DFMO treatment and HSV-TK/GCV gene therapy (**I**). The results did not reveal any synergistic effect; the cytotoxicity with all schedules was not altered significantly compared to the control cells that received only GCV. Based on these results it is concluded that polyamine deprivation as such is not sufficient to enhance the cytotoxic effect of HSV-TK/GCV gene therapy. This raises one question, why did DFMO cause synergism and these other treatments prove to be ineffective? DFMO was able to induce prolongation in S phase cells in conjunction with polyamine depletion and this phenomenon was not observed when DENSPM was used. Although the prolonged S phase may not be the only reason for the observed synergy with DFMO, it is likely that the extended cell cycle effect is the key contributing factor to the positive results when polyamine deprivation was induced with this drug.

In the initial study (**I**) we tested the effect of a polyamine independent approach, serum depletion, on the cell cycle and enhancement of HSV-TK/GCV cytotoxicity. A moderate synergistic effect was detected 10% enhancement with serum deprivation compared to 20% with DFMO (**I**). Since serum depletion as a method is not applicable to animal models or humans and the S phase peak was very transient, we selected a variety of drugs (listed in table 5, page 38.) that are known to arrest the cell cycle at defined points. To characterize the cell cycle effects caused by 24h incubation with the drugs, cells were monitored at six time points following drug removal (**IV**, Fig 3.). One common feature in both cell lines and with all the drugs, except lovastatin, was the rapid increase in the number of cells in the S phase after drug removal. However, this increase was more transient nature when compared to the results obtained with DFMO (**I**, Table II.) or even with serum deprivation (**I**, Table IV).

All drugs were tested for their usefulness in combination treatment with HSV-TK/GCV. It is likely that as a consequence of the transient cell cycle interference induced by aphidicolin, hydroxyurea (HU), mimosin and resveratrol, only a moderate enhancement could be observed. In the 9L cell line, with hydroxyurea and lovastatin, using treatment schedule 2, (GCV added 12h prior to the drug treatment), a marked enhancement without cytotoxic effects to cell growth was

detected (**IV**, Figure 4.). Both HU and lovastatin have previously been described as enhancers of HSV-TK/GCV therapy but we could not replicate these claims (Boucher et al., 2002; Touraine et al., 1998b). Also the possible induction of apoptosis caused by drugs used may have shortened the life span of the HSV-TK positive cells and therefore the drugs actually reduced the effect of the bystander effect. Induction of the bystander effect by many chemicals has been investigated as a strategy to find compounds which can enhance the bystander effect with the HSV-TK/GCV approach. For example, lovastatin which induces an increase in gap junctional communication, has yielded increased cytotoxicity of the HSV-TK/GCV treatment (Touraine et al., 1998b). It is possible that some of the drugs we used may have actually decreased gap junctional communication, as was shown in case of reduction of ODC with DFMO (Shore et al., 2001). This effect of DFMO in our experiments did not disturb the enhancing effect on suicide gene therapy and as a consequence we doubt that the gap junctions are the only mediators of the bystander effect. On the other hand, Shore et al. showed also an increase of gap junctional communication following the increase in ODC activity. It is possible that after DFMO treatment -induced block of ODC and the following release, there could be an elevated ODC activity and consequently an activation of gap junctional communication in parallel to the initiation of GCV treatment.

The results of cell cycle manipulation with a number of drugs (**IV**) and studies with DFMO (**I, III**) together with recent findings of antiapoptotic features of polyamine depletion that can provide enhancement of the bystander effect, indicate that further investigations with DFMO induced enhancement of HSV-TK/GCV treatment are warranted. The next decades will reveal the true effectiveness of suicide gene therapy and its modifications in the treatment of cancer patients. Currently, it is important to continue to study this technique to find new ways to increase its clinical efficacy.

7 SUMMARY AND CONCLUSIONS

The aim of this thesis was to evaluate the potential of HSV-TK/GCV cancer gene therapy enhancement by interfering with the polyamine homeostasis and the cell cycle.

1. Enhancement of HSV-TK/GCV cytotoxicity was possible with inhibition of polyamine biosynthesis with DFMO *in vitro*. This was due to DFMO's parallel influence on the cellular polyamine pools and cell cycle phase distribution. Moreover, serum depletion was found to be a weaker enhancer of HSV-TK/GCV therapy.

2. The TK-GFP fusion gene appeared to be a versatile tool for studying the features of HSV-TK/GCV in animal models. The experiments revealed the different fates of the fusion protein, depending on the animal model used. A strong immune response resulted in a reduction of TK-GFP containing cells in immunocompetent animals. However, this was not seen in an immunodeficient mouse model.

3. Combination treatment of HSV-TK positive tumors with DFMO and GCV yielded more significant tumor suppression than achieved with GCV alone. In view of the demonstrated clinical utility of both treatment forms, DFMO and HSV-TK/GCV combination holds excellent promise for progression into clinical trials.

4. Reduction of polyamine levels without the cell cycle effects was not sufficient to enhance the cytotoxic effect of HSV-TK/GCV gene therapy. Furthermore, drug-induced cell cycle phase synchronization, and the consequent increase in the number of cells in the S phase to be insufficient to achieve the enhancement.

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ORIGINAL PUBLICATIONS

I – IV

I

POLYAMINE BIOSYNTHESIS INHIBITION ENHANCES HSV-1 THYMIDINE KINASE/GANCICLOVIR-MEDIATED CYTOTOXICITY IN TUMOR CELLS

Pasanen T., Karppinen A., Alhonen L., Jänne J. and Wahlfors J.

International Journal of Cancer (2003) 104: 380-388.

II

TK-GFP FUSION GENE VIRUS VECTORS AS TOOLS FOR STUDYING THE FEATURES OF HSV-TK/GANCICLOVIR CANCER GENE THERAPY *IN VIVO*

Pasanen T., Hakkarainen T., Timonen P., Parkkinen J.,
Tenhunen A., Loimas S. and Wahlfors J.

International Journal of Molecular Medicine (2003)12:525-31

III

IN VIVO ENHANCEMENT OF HERPES SIMPLEX VIRUS THYMIDINE KINASE/GANCICLOVIR CANCER GENE THERAPY WITH POLYAMINE BIOSYNTHESIS INHIBITION

Wahlfors T., Hakkarainen T., Jänne J., Alhonen L., and Wahlfors J.

International Journal of Cancer (2006)

IV

POLYAMINE DEPLETION AND CELL CYCLE MANIPULATION IN COMBINATION WITH HSV THYMIDINE KINASE/GANCICLOVIR CANCER GENE THERAPY

Wahlfors T., Karppinen A., Jänne J., Alhonen L. and Wahlfors J.

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