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Review Article

Genetic prodrug activation therapy: a novel treatment for cancer

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CONTENTS

Introduction

Improvement in our understanding of the molecular basis of tumorogenesis has presented an exciting opportunity to utilize these genetic elements and their products as targets for the treatment and ultimately long-term prevention of cancer. The gene families involved in carcinogenesis include dominant oncogenes and tumor suppressor genes that together form a dynamic interplay constraining cell proliferation (1). Targeting the genetic basis of carcinogenesis has led to the development of gene therapy strategies that potentially allow selective destruction of tumor cells by altering or replacing abnormal transformation-related genes in cancer cells or by inducing a specific sensitivity to a prodrug (2).

In the following review we discuss genetic prodrug activation therapy (GPAT) which utilizes the transcriptional differences between normal and neoplastic cells to drive the selective expression of a metabolic suicide gene able to confer sensitivity to a prodrug.

Metabolic suicide gene/prodrug systems

Genetic prodrug activation therapy (GPAT) can be described as the intracellular conversion of a nontoxic prodrug to a toxic molecule by enzymes not normally present in mammalian cells. The enzymes employed for GPAT originate from bacteria, viruses and fungi where they control unique metabolic processes. These proteins have been the major targets for the development of selective prodrugs to treat infection for many years. Such prodrugs are lethal for the invading microbe but harmless to the host cell that lacks the enzymes necessary for the prodrug's modification. If the genes encoding these enzymes are transiently expressed in the host cell, then it too will exhibit the sensitive phenotype. Treatment with the appropriate prodrug will confine toxicity to those cells expressing the enzyme and the microenvironment surrounding them while normal cells will remain unaffected (3). More recently, mammalian genes encoding enzymes that are tissue-specific or expressed only under certain conditions have also been exploited for use in GPAT systems (4).

Choosing the correct enzyme is the most important consideration, as appropriate prodrugs can be synthesized for almost any enzyme. Theoretically, the most suitable enzyme would be a simple low-molecular weight, monomeric protein with no requirement for posttranslational modification. More complex enzymes may not form the correct protein conformation in different species and as a result be inert. In addition, the enzyme should activate the prodrug rapidly (high K_{cat}) and function at low concentrations of the substrate (low K_m). The only prerequiste for the prodrug is that it should be approximately two orders of magnitude less cytotoxic than the active metabolite (5).

Several enzyme/prodrug systems have been reported (Table I) and the prototypes are described below.

Herpes simplex thymidine kinase

Herpes simplex virus thymidine kinase (HSV-TK) is distinct from cellular thymidine kinases and is the target for the guanosine analog prodrugs, ganciclovir and acyclovir (6). HSV-TK metabolizes ganciclovir to its monophosphate form and this is the rate-limiting step. The monophosphorylated prodrug is then further metabolized to its diphosphate form by cellular guanylate kinase (7). Conversion to the cytotoxic ganciclovir-triphosphate

Table I: Enzyme/prodrug systems currently under investigation.

Enzyme	Prodrug	Drug
Cytosine deaminase	5-Fluorocytosine	5-Fluorouracil
Viral thymidine kinase	Ganciclovir 6-Methoxypurine arabinonucleoside (araM)	Ganciclovir triphosphate Adenine arabinonucleoside Triphosphate (araTP)
Nitroreductase	CB-1954 4-Nitrobenzyloxycarbonyl derivatives	5-(Aziridin-1-yl)-4-hydroxyl amino-2-nitrobenzamide) Actinomycin D Mitomycin C
Cytochrome P450	Cyclophosphamide Ifosfamide	Phosphoamide mustard (+acrolein)
Alkaline phosphatase	Doxorubicin/etoposide/mitomycin	Doxorubicin/etoposide/ mitomycin-phosphate
Xanthine oxidase	Xanthine	Oxygen radicals
Carboxypeptidase A Carboxypeptidase G2	Methotrexate-alanine Benzoic acid mustard gluconuride	Methotrexate Benzoic acid mustard
β-Lactamase	Cephalosp-mustard-carbamate	Nitrogen mustard
β-Glucosidase	Amygdalin	Cyanide
β-Glucuronidase	Adriamycin/daunorubicin/ epirubicin-gluconuride	Adriamycin/daunorubicin/epirubicin
Linamarase	Amygdalin	Cyanide

is then completed by phosphoglycerate kinase, pyruvate kinase and phosphoenolpyruvate kinase. The triphosphate form of the prodrug functions by inhibiting α-DNA polymerase (8) and is incorporated into DNA resulting in chain termination during replication (9). There are potential disadvantages with this enzyme/prodrug system. First of all, the drug is an S-phase specific agent, suggesting that it is only effective on cycling nonresting cells within a tumor. As a consequence, the prodrug must be administered continuously until all the cells enter S-phase (5). However, this view has been challenged by a number of researchers who have reported inducible ablation in tissues with low mitotic indices (10-13). The actual mode of action of the activated prodrug in these cases is unclear, although some evidence from nonproliferating thyrocytes suggests it occurs via p53-independent apoptosis (13). Secondly, complications could theoretically result from the active drug being a triphosphate with poor diffusion properties and as a result providing a decreased bystander effect (discussed in more detail in a subsequent section). However, observations *in vitro* and *in vivo* suggest that the bystander effect is caused by metabolic cooperation and also has an immune component (14-16).

Varicella zoster thymidine kinase

Studies have demonstrated that the prodrug 6-methoxypurine arabinonucleoside (9-(β-D-arabinofuranosyl)-6-methoxy-9*H*-purine (araM)) is a good substrate for varicella zoster virus thymidine kinase but poor for the three major cellular kinases. araM is monophosphorylated by VZV-TK to araM monophosphate which is further metabolized by cellular enzymes AMP deaminase, adenylsuccinate synthetase lyase, AMP kinase and nucleoside diphosphate kinase to its cytotoxic form adenine arabinonucleoside triphosphate (araATP) (17).

Cytosine deaminase

Cytosine deaminase is found in bacteria and fungi where it is activated in response to nutritional stress deaminating cytosine to uracil (18). As a result, this enzyme became the target for drug design to fight such infections. 5-Fluorocytosine (5-FC) was developed and shown to be effectively deaminated to 5-fluorouracil (5-FU) by cytosine deaminase. Further metabolism of 5-FU to 5-fluorouridine 5'-triphosphate and 5-fluoro-2'-deoxyuridine 5'-monophosphate results in cell death by affecting RNA and DNA synthesis. 5-FU has an advantage over ganciclovir-triphosphate, as it is not phase-specific but is proliferation-dependent. One disadvantage is that high dose levels are required to achieve cytotoxicity, and there have been some reports of resistance to the toxic metabolite. For instance, Harris *et al.* (19) demonstrated *in vitro* that 5-FU applied directly to a panel of breast and pancreatic tumor cell lines resulted in approximately 5% survival even after 7 days exposure to 130 µg/ml. It was postulated that this may have been due to defects in downstream metabolism. Huber *et al.* (20) observed that the cytotoxic effects of 5-FC and constitutively expressed cytosine deaminase provided significantly better results *in vivo* than those achieved *in vitro* with 5-FU and colon cancer cells (WiDr) which are exquisitely sensitive to 5-FU.

Nitroreductase

Nitroreductase (NTR) is a monomeric enzyme that is able to convert nontoxic monofunctional alkalating agents to their difunctional forms, which are 4 orders of magnitude more cytotoxic (21). *Escherichia coli* NTR has been used to metabolize the prodrug 5-(aziridin-l-yl)-2,4-dinitrobenzarnide (in the presence of cellular NADH or

NADPH acting as the reductant) to 5-(aziridin-1-yl)-4-(hydroxyamino)-2-nitrobenzarnide, which is acetylated to 5-(aziridin-l-yl)-4-(acetylamino)-2-nitrobenzamide. This then acts by cross-linking cellular DNA resulting in apoptosis. One of the advantages of this system is the availability of a variety of cytotoxic agents that may be adapted to prodrugs for this enzyme. Recently, NTR has been used in transgenic mouse models expressed under the control of the ovine β-globin promoter in luminal cells of the mammary gland. Treatment with CB-1954 led to selective killing of the luminal cells but had no effect on closely associated myoepithelial cells (22). This system offers advantages over both HSV-TK and CD as it does not require cell proliferation.

Carboxypeptidase G2

The bacterial enzyme carboxypeptidase G2, which activates the prodrugs benzoic acid mustard glutamates to benzoic acid mustards, has also been considered for GPAT. However, the major drawback with this system is the high affinity for folates, resulting in rapid depletion and cellular death. This is a potential problem as the success of GPAT (with the current gene delivery vehicles) relies on the bystander effect which would be nonexistent if the cells are killed as a result of the enzyme alone (5).

Cytochrome P450

Recently, Chen and Waxman (4) reported the use of rat cytochrome P450 2B1 (CYP 2B1) as a suicide enzyme in combination with the oxazaphosphorine prodrug cyclophosphamide as a potential treatment of cancer. Cyclophosphamide is activated by cytochrome P450 via a 4-hydroxylation reaction to the active components phosphoramide mustard and acrolein, which alkalate DNA and proteins. Cytochrome P450s are poorly expressed in tumor cells but are abundant in the liver. As a consequence, in the past treatment of tumors with oxazaphosphorine had little effect on tumor burden, but due to overexpression of cytochrome P450s in the liver, metabolism of the prodrug led to unacceptibly high systemic toxicity. Chen and Waxman (4) demonstrated that 9L gliosarcoma cells transfected *in vitro* with CYP 2B1 were sensitized to the prodrug cyclophosphamide compared to the wild-type cells. Suprisingly, when these cells were implanted into a mouse model as xenografts and treated with the prodrug, the tumors containing CYP 2B1 regressed compared to the wild-type. This was the opposite of the predicted result which postulated that there would be little difference between the CYP 2B1 expressing tumors and the wild-type as the prodrug would be activated by the liver metabolism. It was concluded that the local activation of the prodrug was superior to the liver and that acrolein was sensitizing the cells to the mustard. A similar study by Wei *et al.* (23) demonstrated that *in vivo* xenografts expressing CYP 2B1 regressed compared to wild-type cells. They also showed that the toxic metabolites were freely diffusible giving rise to a significant bystander effect.

With such evidence it appears that some mammalian enzymes may indeed provide viable alternatives for the GPAT systems.

Targeting strategies for genetic prodrug activation therapy

Two targeting strategies (*i.e.*, transduction and transcriptional) have been developed for GPAT to ensure that expression of a suicide gene is restricted to the malignant target tissue (Fig. 1).

Transduction targeting, which relies on the preferential delivery of genes to actively dividing cells by means of a viral vector, was first described by Molten (24) who showed that murine cells transduced with HSV-TK were sensitized to ganciclovir. The murine retroviral vectors are the most commonly used, but although potentially powerful, this targeting system is restricted to tumor cells in organs where the resident tissues are generally nonproliferative.

Transcriptional targeting places the therapeutic gene under the control of transcriptional regulatory elements that possess binding sites for tissue or tumor restricted *trans*-acting factors (25). As a consequence, for such a system to be effective, the regulatory elements of the promoter/enhancer need to be fully characterized (26).

These two methods can be combined to provide an improved targeting system, but it should be pointed out that to date no gene transfer system has been shown to be 100% reliable (2) and each system has its own disadvantages which are described in more detail in the following sections.

Transduction targeting

One of the first and most interesting reports describing transduction targeting of a suicide gene was made by Culver *et al.* (27) for the treatment of intracerebral gliomas. Retroviral vector producer lines (VPC) were used to deliver replication-defective retroviruses carrying the HSV-TK gene into rat brains containing small intracerebral gliomas. Animals were then treated systematically with ganciclovir over a 5-day period. Postmortem analysis showed that 75% of the tumors had regressed in the treated animals compared to untreated controls. One of the major advantages of this system is the unique delivery of the retrovirus/suicide construct. By depositing vector producing cells, a higher circulating titer of the virus can be expected in the 7-day interval prior to treatment with ganciclovir, which in turn should improve the efficacy of cell transduction.

Other studies using VPC have also been described for hepatocellular carcinoma and stage III ovarian cancer

(28). Caruso *et al.* (29) showed that direct injection of VPC (secreting a retroviral vector expressing the HSV-TK) into hepatic tumors in rats produced significant anti-tumor effects without causing toxicity to the normal surrounding hepatocytes (which have a high baseline proliferative rate). At present several clinical trials have been designed to evaluate this system (30, 31).

Similar strategies employing adenovirus delivery systems have also been described. Hirschowitz *et al.* (32) recently reported the successful treatment of colonic carcinomas using the cytosine deaminase suicide gene, constitutively expressed under control of the cytomeglavirus (CMV) promoter. The chimearic gene (AdCMV.CD) was delivered to HT29 colon carcinoma cells both *in vitro* and *in vivo* using a replication defective adenovirus. The AdCMV.CD vector suppressed growth of the HT29 cells *in vitro* in the presence of 5-FC in a dose-dependent manner. When the AdCMV.CD was directly injected into established subcutaneous HT29 tumors in nude mice receiving 5-FC, there was a 4-fold reduction in tumor burden after 15 days and a 5-fold reduction after 28 days compared to control animals.

Hwang *et al.* (33) described the use of the HSV-TK gene under control of the Rous sarcoma virus long terminal repeat (LTR) delivered by an adenovirus vector (Ad.RSV.TK) for treatment of malignant mesothelioma. REN mesothelioma cells transduced with Ad.RSV.TK were demonstrated to be 100- to 1000-fold more sensitive to ganciclovir compared to nontransduced REN cells (34). *In vivo* studies using a SCID mouse model of mesothelioma showed that 9 out of 10 animals treated with Ad.RSV.TK and ganciclovir had a decreased tumor burden, while 19 of the 20 control animals had large amounts of tumor mass in the peritoneal cavity.

Although these retroviral and adenoviral targeting systems appear to be effective, their reliance on constitutive promoters to drive expression of the therapeutic gene means that use of these vectors increases the risk of normal cells being transduced. Therefore, vectors that can target specific cell types or retain the virus in the vicinity of the tumor in order to reduce their systemic spread are required for effective cancer gene therapy. Hence, viruses with modified tropisms are being investigated to improve the chances of site-specific targeting.

Modification of retroviral and adenoviral tropism

Retroviral infection is initiated by the attachment of the virus particle to specific cell surface receptors mediated by the surface subunit (SU) of the retroviral envelope protein (encoded by the *env* gene). This has become the focus for many researchers interested in modifying the tropism of these viruses (35). These strategies are based

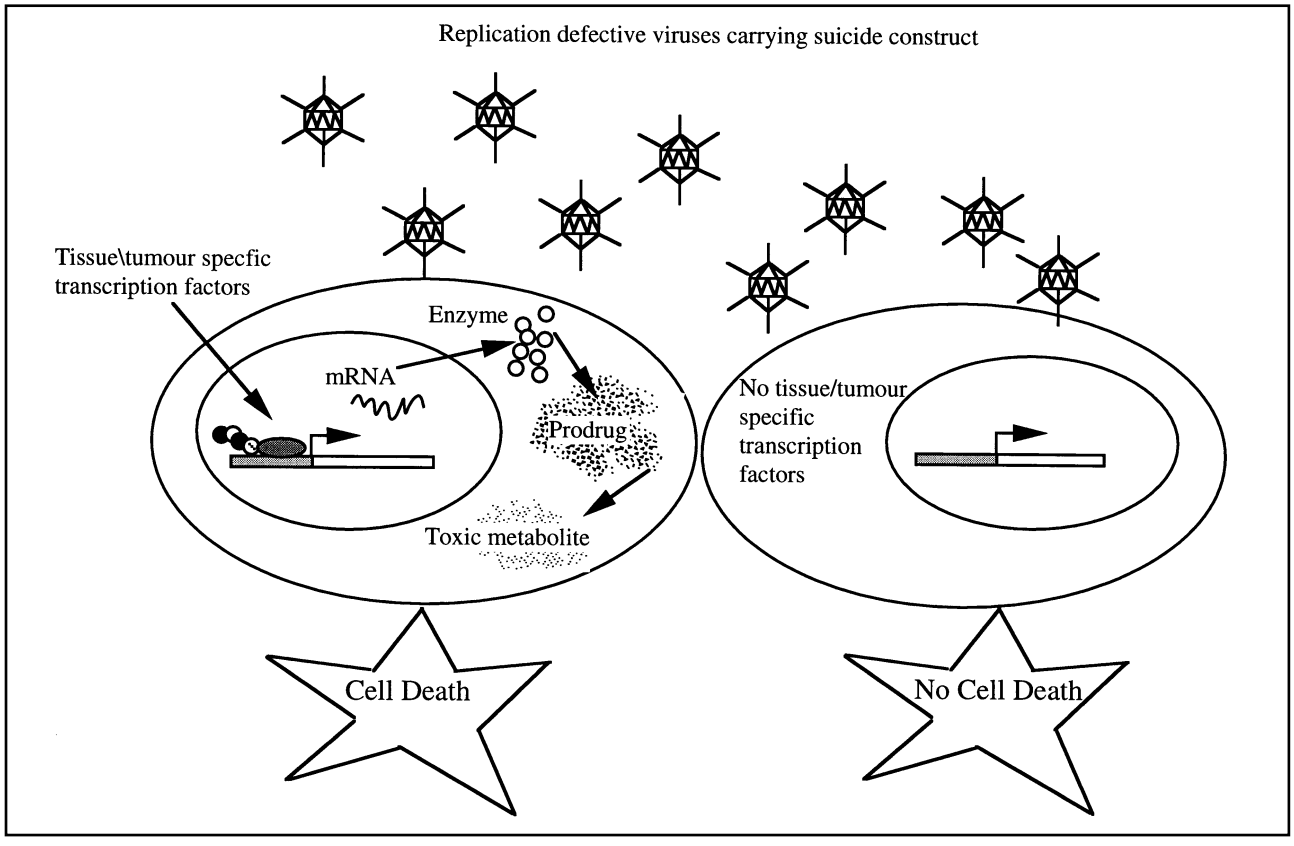

Fig. 1. Schematic diagram representing genetic prodrug activation therapy.

on the introduction of binding sites specific for receptors on the surface of target cells. Russell *et al.* (36) have reported the successful fusion of a gene encoding a single chain Fv antibody fragment (directed against a cell surface antigen) to an ecotropic mouse moloney leukemia virus (MMLV) envelope gene. The hybrid retroviruses produced were able to bind antigen by the single chain Fv but were unable to infect cells expressing the antigen. However, studies by Chu *et al.* (37) have demonstrated that fusion of a single chain Fv to the 3' end of the spleen necrosis virus *env* gene did result in infectivity of antigen-presenting cells but only at an efficiency of 2% compared to the wild-type virus.

Pioneering work has been carried out by Valsesia-Wittmann *et al.* (38), who successfully altered the tropism of avian leukosis virus (ALV) by inserting a 16 amino acid sequence containing an RGD motif into the envelope protein. The hybrid viruses were not only able to infect cells expressing the ALV receptor but also mammalian cells expressing an RGD motif. However, as reported by Chu *et al.* (37), the infection level was poor. Cosset *et al.* (39) have reported the insertion of sequences for two different cell surface molecules, *i.e.*, ligands for the epidermal growth factor receptor (EGFR) and Ram-1 phosphate transporter (a receptor used by amphotropic murine leukemia virus (MLV-A)). Both hybrid virions recognized their respective receptors, but the EGF chimeras were completely noninfectious and those expressing Ram-1 had only a fraction of the efficacy of the wild-type virus. It was further postulated that the noninfectivity of the EGF hybrid was a result of the ligand acting as an agonist for targeted receptors, so that EGFR-bound virus was taken up by lysosomes leading to abortive infections.

Further studies (40) examining the low infectivity for the Ram-1 MMLV hybrid indicated that this was due to poor fusion between the viral and cellular membrane leading to low transduction signals. Studies examining the positional effect of the Ram-1 domain on infectivity showed that fusion to codon 7 of the surface protein (SU encoded by *env*) resulted in only low level infectivity, while hybrid viruses where Ram-1 was fused to either codon 6 or 1 of the SU showed an increase of 30- or 100-fold infectivity, respectively. It was concluded that the correct interdomain spacing between foreign sequences and the surface protein has a significant effect on virus infectivity.

The most exciting report to date of a targeted retrovirus system has been made by Nilson *et al.* (41). They have developed a targeting system which allows the MMLV to use its natural cell receptor-mediated entry mechanism in conjunction with a hybrid receptor. When the EGF ligand was fused to codon 1 of the SU domain upstream of a factor Xa protease cleavage site, the resulting virus hybrid was capable of targeting the EGF receptor but no infectivity was noted. However, upon the addition of factor Xa the virus infectivity increased more than 100-fold. The mechanism allows the virus to be targeted to, and accumulated at, specific cells via the novel receptor binding activity followed by cleavage with a site-specific protease allowing virus internalization through its natural receptor route. By modifying the system further and incorporating metalloprotease or serine protease cleavage sites into the *env* gene (both upregulated in tumors), it would produce an extremely effective targeting system (that would not act as an agonist for receptor-mediated pathways) for the delivery of therapeutic genes to tumors.

Very few studies on targeted adenoviral vectors have been reported. Infection of cells by adenovirus particles takes place in two stages. Initially, the fiber binds to an as yet uncharacterized cell receptor followed by the interaction of the RGD sequences encoded on the penton base with cell surface integrins triggering internalization. One of the major problems associated with the use of adenovirus vectors is the ubiquitous nature of the fiber protein receptor making targeting difficult. However, attempts have been made to redirect binding specificity by conjugating molecules such as part of the gastrin-releasing peptide to the carboxy terminus of the human adenovirus type 5 fiber protein (42).

Most of the studies have concentrated on the modification of the penton base which has five copies of an RGD motif (integrin receptor binding motif) allowing interaction with the vitronectin binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Modifications of the penton base have allowed other integrins to be targeted. For example, replacing the RGD motif with LDV directed binding of recombinant adenoviruses to integrin $\alpha_4\beta_1$. A further adenovirus construct was engineered by removing the peptide selectivity for binding to $\alpha_v\beta_5$ but retaining it for $\alpha_v\beta_3$ (43). With further refinement of this approach, selective delivery via a range of cancer-associated molecules could be achieved.

Transcriptional targeting

Transcriptional targeting relies on tissue or tumor-specific regulatory sequences to drive the expression of the therapeutic gene only in those cells that contain transcription factors able to activate the promoter elements (Table II). Several examples of this system are described below.

Huber *et al.* (44) used the α-fetoprotein or liver-associated albumin transcriptional elements to prepare artificial chimeric genes that generated either hepatoma-specific or liver-specific expression of VZV-TK, respectively. The chimeric genes were delivered *in vitro* by a replication incompetent retrovirus and, although the initial results were exciting, a similar recombinant retrovirus carrying the construct failed to express the gene in transgenic mice. This was presumed to be due to silencing of the retroviral sequences (45).

Many human melanomas synthesize melanin which is produced exclusively by melanocytes. The pathway involves hydroxylation of tyrosine by the tyrosinase enzyme which is highly expressed by melanocytes (46). As a consequence, the tyrosinase transcriptional elements have provided a target for the development of a

Table II: Gene transcriptional elements that could be exploited for GPAT systems.

Gene	Tumor or tissue type
ERBB2	Breast, pancreatic and gastric tumors
ERBB3	Breast and gastrointestinal cancers
ERBB4	Breast and gastrointestinal cancers
MUC1	Breast, pancreatic duct tumors
Carcinoembryonic antigen (CEA)	Colorectal, pancreatic and gastric tumors
Bombesin	Small cell lung carcinoma
DOPA decarboxylase	Small cell lung carcinoma
Neuron-specific enolase	Small cell lung carcinoma
Tyrosinase	Melanoma
Tyrosinase-related protein (TRP-1)	Melanoma
Prostate-specific antigen	Prostate cancer
Secretory leukopeptidase inhibitor	Lung
Thyroglobin	Follicular carcinoma of the thyroid
Insulin	Insulinoma
11β-Hydroxylase	Adrenocortical carcinoma
α-Fetoprotein	Hepatocellular carcinoma

GPAT strategy to treat melanoma. Vile and Hart (47, 48) placed a β-galactosidase gene under the transcriptional control of the tryrosinase (TRP-1) promoter and demonstrated tissue-specific expression of the reporter gene in B16 melanoma cells but not in the control cell line NIH 3T3 fibroblasts. They went on to replace the reporter gene with HSV-TK. Murine melanoma cells expressing HSV-TK under control of the TRP-1 promoter were injected into immunocompetent syngeneic mice. The mice were then either given ganciclovir (150 mg/kg day) or vehichle treated. The control animals (27 of 28) showed marked tumor burden, while 29 of the 30 animals treated with ganciclovir demonstrated tumor regression.

One problem that remains to be addressed with this system is the expression of tyrosinase in other cell types, *e.g.*, spinal ganglia, Schwann cells and astrocytes, which could result in nonspecific targeting of the suicide construct after systemic delivery.

The proto-oncogene ERBB2 has been shown to be overexpressed in a number of breast and pancreatic carcinomas (49). The transcriptional upregulation of ERBB2 in breast cancer has been associated with the interaction of a positively acting transcription factor, with a response element in the proximal part of the promoter (50). The tumor-specific transcription factor identified is thought to be a member of the AP2 family (51). Harris *et al.* (19) have used a 500 bp fragment of the proximal promoter containing the AP2 site to drive tumor-specific expression of a CD suicide gene in a panel of pancreatic and breast tumor cell lines, with differing ERBB2 expression patterns. The level of CD expression and subsequent cell death (in the presence of 5-FC) was directly proportional to the ERBB2 status of the cell.

One of the most exciting developments in GPAT to date is the use of chimeric transcriptional elements to improve tissue/tumor-specific targeting. Richards *et al.* (52, 53) have described the use of the carinoembryonic antigen (CEA). This is a tumor-associated cell surface molecule (54, 55) which is expressed in normal tissue but is transcriptionally upregulated in many colorectal and lung cancers, making it an ideal target for GPAT. To determine the *cis*-acting transcriptional regulatory sequences of the CEA gene, Richards *et al.* (53) took fragments of the 5' untranslated region (up to 14.5 kb upstream of the transcriptional start) and used them to drive expression of a luciferase reporter gene in CEA-positive and CEA-negative expressing cell lines. The first generation of constructs (10,800 bp to 299 bp of the 5' sequence) demonstrated a 5- to 10-fold increase in tissue-specific expression in CEA-positive compared to CEA-negative cell lines. However, further analysis of the shorter constructs revealed a strong *cis*-acting factor. By combining multimers of this sequence together with two upstream elements, the tissue-specific expression was reported to be 2-4 times higher than that of an equivalent luciferase reporter construct under control of the SV40 promoter/enhancer. The chimeric-CEA construct was used to express the CD gene *in vivo*. NCI H508 cells (CEA-positive) were transduced with the construct and used together with nontransduced NCI H508 cells to produce tumor xenografts in SCID mice. The animals were then treated once a day with 500 mg/kg 5-FC over a 3-week period. Mice containing the CEA-CD construct showed no significant tumor burden compared to the control mice 20 days after cessation of the prodrug.

Osaki *et al.* (56) used a similar principle for the potential treatment of lung cancer. In this study the CEA gene was used to drive expression of the HSV-TK gene. CEA expressing lung cancer cells were transduced with the chimeric construct and used to produce xenografts in nude mice. Animals were subsequently treated with ganciclovir and demonstrated a significant reduction in tumor burden compared to the control mice.

The Myc binding sequence has also been proposed as a potential candidate for tumor-specific expression of suicide genes (57). The Myc-Max oncoprotein complex (which is overexpressed in a number of tumors) has the ability to bind the sequence CACGTG resulting in transcriptional activity. An artificial chimeric gene was made consisting of four repeats of the Myc sequence upstream of a minimal promoter driving expression of a luciferase reporter gene. When transfected into Myc-positive cells

the expression of the reporter gene was 4- to 6-fold higher than in Myc-negative cells (57). *In vivo* studies demonstrated that nude mice with established Myc-positive tumors showed a 50% decrease in tumor burden when treated with a *Pseudomonas* enterotoxin suicide gene. Kumagai *et al.* (58) reported similar results using an HSV-TK suicide system.

Su *et al.* (59) recently reported the use of a replication incompetent adeno-associated virus to deliver a chimeric gene composed of the albumin promoter element joined to the α-fetoprotein enhancer, to drive expression of the HSV-TK gene. Delivery of this minigene resulted in specific killing of α-fetoprotein and albumin-positive hepatocellular carcinoma cells, while transduced albumin and α-fetoprotein-negative cells were not succeptible to ganciclovir.

Transcriptional targeting of a recombinant adenovirus to human and murine melanoma cells, using elements of the tyrosinase promoter/enhancer, has been reported by Siders *et al.* (60). They demonstrated that a 2500 bp fragment of the 5' regulatory region, used to drive expression of a β-galactosidase reporter gene in murine melanoma cells, resulted in very low transcriptional activity. However, a 209 bp fragment of the tyrosinase promoter, coupled to two tandem repeats of an upstream enhancer element, resulted in high levels of tissue-specific expression. The cassette was transferred to the E1 region of a recombinant adenovirus, which when used to transduce melanoma cells maintained its transcriptional specificity and, more interestingly, was found to be extremely active in human melanoma cells (reportedly exceeding the transcriptional activity of a CMV β-galactosidase control construct).

From the *in vitro* and *in vivo* data described, the ability to target tumors at the transcriptional level is clearly encouraging. However, there have been reports of difficulties in maintaining tissue specificity and expression of therapeutic genes both *in vitro* and *in vivo*. For example, Ring *et al.* (61) demonstrated that insertion of an HSV-TK gene (under control of the ERBB2 promoter) into either the E1 or E3 regions of recombinant adenoviruses resulted in loss of tissue specificity. In contrast, the same cassette delivered by a recombinant retrovirus maintained lineage-specific expression. Similarly, as discussed previously, Richards and Huber (45) reported difficulties in transferring their α-fetoprotein-VZV suicide construct into an *in vivo* model using a retroviral delivery system.

Vile *et al*. (62) compared the properties of different retroviral vectors containing the murine tyrosinase promoter driving the expression of HSV-TK or IL-2 genes. They concluded that the degree of tissue-specific expression from the internal tyrosinase promoter depended on the molecular design of the vector, and that loss of specificity was most probably the result of juxtaposed internal promoters within the provirus. Another drawback associated with recombinant retroviruses are the low viral titers which have been associated with insertion of large minigenes, often in the opposite orientation to the viral LTR (63).

Vile *et al.* (63) have demonstrated a strategy to alleviate such problems by replacing the Mo-MLV enhancer (located in the LTR) with the murine tryrosinase promoter/enhancer element. They demonstrated that the hybrid tyrosinase-LTR driving IL-2 expression was basal in nonmelanoma cell lines compared to melanoma cell lines, which expressed high levels of IL-2. They concluded that no promoter interference was evident.

Further advances in the design/development of "tailor-made" transcriptional elements, together with improvements in viral delivery systems, may provide GPAT as a feasible alternative for cancer treatment.

Bystander effect

With the vector systems currently available, it is impossible to deliver therapeutic genes to every cell. Also, use of retrovirus-mediated gene transfer results in random integration of the inserted genes within the tumor population. As a consequence, cells will have widely varying levels of transgene expression. One of the most interesting paradoxes is that not all tumor cells need to be transduced with a suicide construct to provide effective cell killing. The process whereby nontransduced cells in a mixed population die in the presence of a given prodrug has been termed "bystander killing".

The first report of the bystander effect demonstrated that in mixed populations of HSV-TK transduced and nontransduced cells, plated at low dilutions and treated with ganciclovir, only the HSV-TK expressing cells were killed. In contrast, when the cell mixture was plated at high density, nearly all the cells (HSV-TK-positive and -negative) died. It was postulated that some form of metabolic cooperation between juxtaposed cells allowed transport of the active metabolite (24). The bystander effect was also demonstrated *in vitro* using a human fibrosarcoma model. Cells were either transduced with a β-galactosidase reporter gene (ganciclovir-resistant) or HSV-TK (ganciclovir sensitive). The cells were co-cultured at different dilutions in the presence of ganciclovir. At confluence both cell lines died, while at a 10-fold lower density only HSV-TK transduced cells died and minimal effect on the β-galactosidase cell survival was noted (14).

Further studies using [^{3}H]-ganciclovir showed incorporation of the labeled drug into the nuclei of HSV-TK transduced cells, but not those expressing β-galactosidase. However, when both cell populations were cultured at high densities, the labeled ganciclovir was incorporated into the nuclei of both cell populations. It was surmised that physical contact between the adjacent cells allowed "metabolic cooperation". This is a process allowing low molecular weight (<1000) molecules to pass between cells via gap junctions (these are small hexameric structures in the cell membrane which form part of a cell to cell communication network). It is feasible that phosphorylated ganciclovir, which has a molecular weight of approximately 300, may enter adjacent cells via these gap junctions and cause cell death (14).

Other *in vitro* studies have suggested that the bystander effect is the result of phagocytosis of apoptitic vesicles containing HSV-TK and ganciclovir from dying cells (16).

In vivo studies using a retroviral vector to deliver the HSV-TK gene to rat gliomas have also demonstrateted a strong bystander effect. In this case, analysis of the tumors showed that only 10-70% of cells expressed the transgene and yet significant or complete tumor regression was noted (2). Recently, immune responses have been implicated in mediating the bystander effect (15, 64). Mullen *et al.* (64) used two tumor cell lines, 102 (nonmetastatic fibrosarcomas) and 38 (nonmetastatic colon adenocarcinoma), transduced with a retroviral vector carrying the CD gene to produce xenografts in a mouse model. The mice treated with 5-FC showed complete tumor suppression compared to untreated control animals. Interestingly, when animals previously treated with 5-FC were challenged with wild-type tumor cells, they exhibited a significant type-specific resistance. This was attributed to a T-cell-mediated response, either as a result of CD-positive cell death leading to improved antigen presentation or the CD protein acting as an immunogen. In a similar study (15), nude mice (T-cell deficient) and immunocompetent mice with established lung metastases (B16 melanoma) were treated intravenously with a recombinant retrovirus containing the HSV-TK gene, under control of the TRP-1 promoter. When given ganciclovir, the immunocompetent mice showed a 90% reduction in tumor burden compared to untreated control animals. However, there was no significant difference in the number of lung metastases in the nude mouse model. This suggested that a T-cell-dependent mechanism was amplifying the therapeutic effect. To confirm this, mice were vaccinated with B16 HSV-TK cells which were either lethally irradiated or allowed to develop *in situ* before being excised. The mice were then challenged with wild-type or HSV-TK transduced B16 cells but no protection was evident. However, when mice treated with B16.HSV-TK were given ganciclovir, a protective response to both wild-type B16 and B16.HSV-TK cells was noted but challenge with an unrelated tumor line offered no protection (15).

Further studies of these models have shown local cytokine release, leading to necrosis of malignant cells and infiltration of immune cells into the tumor. Cytokines released in this way may upregulate the expression of immunomodulatory molecules such as the B7 family and the ICAMs (intracellular adhesion molecules), facilitating changes in the tumor microenvironment from immunoinhibitory to immunostimulant. This in turn may encourage an immune response against the tumor cells (65). Some of these changes have been observed specifically after HSV-TK gene therapy (66). HSV-TK modified cells die through apoptosis, during which they release soluble inflammatory factors such as IL-1. IL-1 causes too weak an immune response to affect the entire tumor load, allowing the tumor to grow to a size that is too large to be eliminated when antitumor immunity develops several weeks later. A method to boost the rather weak inflammatory response may be to combine HSV-TK gene therapy with active immunotherapy by coexpressing IL-2 or IFN-α. IL-2 does not on its own increase the bystander effect but has been associated with development of long-term tumor immunity. However, IFN-α has been shown to enhance the bystander effect (67).

Clearly, further improvements in our understanding of the bystander effect are necessary if we are to exploit this paradigm to improve current GPAT strategies.

Clinical protocols approved for GPAT

Several clinical trials have been approved (Table III), although to date results from these trials are unavailable. The majority of those outlined rely on transductional targeting using either retroviral or adenoviral vectors.

Oldfield *et al.* (30) have treated cerebral gliomas using sterotactic apparatus fixed to the patient's skull to deposit vector producing cells (VPC) (secreting the replication defective retrovirus and HSV-TK gene). Seven days later patients are treated with a twice-daily dose (5 mg/kg) of ganciclovir over 14 days. However, the protocol suffers from several drawbacks. Firstly, the human tumors generally have a lower proliferative rate compared to animal models, therefore the transduction targeting may be less efficient. Secondly, the system relies on a strong bystander effect, and finally, the ratio of VPCs to tumor cells achieved does not match the 100:1 used in the animal model. Even so, tumor regression has been observed in several patients but recurrences are regularly noted (3).

Eck and Alavi (69) are using a similar protocol to treat malignant gliomas. Here, replication incompetent adenoviruses are used to deliver the HSV-TK gene under the control of the Rous sarcoma virus (RSV) LTR. Similarly, Albelda (70) is using the same adenoviral vector to treat mesothelioma characterized by tumors in the pleural space. Patients are grouped and treated with the adenoviral vector (doses range from 10^9 to 10^{12} pfu) followed by intravenous administration of 5 mg/kg of ganciclovir twice daily over 14 days.

Grossman and Woo (71) are treating brain tumors with a replication incompetent adenovirus to deliver an HSV-TK gene under constitutive control of the RSV LTR. Patients receive a primary dose of vector (1×10^8 pfu) followed by a 10 mg/kg/day intravenous dose of ganciclovir over 2 weeks. The effectiveness of the treatment is monitored by MRI and CT scans.

Our laboratory has developed a suicide construct consisting of the CD gene driven by the ERBB2 promoter. Breast tumors are inoculated with up to 400 µg of the plasmid followed by a 48-hour infusion of 5-FC. Several patients have successfully completed the trial and initial results are encouraging.

Future perspectives for GPAT

Although the observations both *in vitro* and *in vivo* look encouraging, there are still many factors that need to

Table III: Current phase I clinical trials approved for genetic prodrug activation therapy.

Principal investigator	Protocol title	Target	Gene transduction	Vector name	Delivery vehicle	No. of patients	Evidence of gene expression	No. and type of response	Adverse reaction
Albelda SM Univ. of Pennsylvania	Gene therapy for malignant mesothelioma with HSV-TK	Malignant mesothelioma cells	*In vivo*	H5.01ORSVTK	Adenovirus	10	Yes	None	Fever, abnormal liver function
Curiel D. Univ. of Alabama	Adenovirus intraperitoneal HSV-TK for ovarian and extra ovarian cancer patients	Ovarian cancer cells	*In vivo*	AdTK	Adenovirus	0	NA	NA	NA
Eck SL Univ. of Pennsylvania	Recombinant adenovirus for treatment of CNS cancer	Glioblastoma/ astrocytoma cells	*In vivo*	H5.01ORSVTK	Adenovirus	2	NA	None	None
Grossman RG Baylor College of Medicine	HSV-TK for CNS tumors	Brain tumor cells	*In vivo*	Adv. RSV-tk	Adenovirus	0	NA	NA	NA
Fetell MR Columbia Presbyterian Med. Center	Stereotactic injection of HSV-TK vector for treatment of recurrent malignant glioma	Glioma cells	*In vivo*	GITKISvNa.7	Retrovirus	2	NA	None	NA
Finocchiaro G Inst. Nazionale Neurologico Milan	Gene therapy of glioblastoma with HSV-TK	Glioblastoma cells	*In vivo*	HSV-TK	Retrovirus	0	NA	NA	NA
Freeman SM Tulane Univ. Med. Center	Treatment of ovarian cancer with a modified HSV-TK cancer vaccine	PA-1 ovarian tumor cells	*Ex vivo*	STK	Retrovirus	14	NA	2CRs	Fever, abdominal pain, nausea
Freeman SM Tulane Univ. Med. Center	Vaccination with HER-2/neu-expressing tumor cells and HSV-TK gene-modified cells	PA-1 ovarian MDA breast cancer cells	*Ex vivo*	STK/B7	Retrovirus	0	NA	NA	NA
Izquierdo M Univ. Autonoma de Madrid	Gene therapy for metastatic melanoma with HSV-TK	Melanoma cells	*In vivo*	ptk zip neo	Retrovirus	9	No	1PR: 1MR	Fever
Klatzmann D Hosp. Pitie Salpetiere	Gene therapy for metastatic melanoma with HSV-TK	Melanoma cells	*In vivo*	pMTK	Retrovirus	7	Yes	NA	None
Klatzmann D Hosp. Pitie Salpetiere	Gene therapy for glioblastoma	Glioblastoma	*In vivo*	pMTK	Retrovirus	13	NA	NA	None
Kun LE St. Judes Childrens Hosp. Memphis	Stereotactic injection of HSV-TK producer cells for progressive or recurrent primary supratentorial pediatric brain tumors	Neoplastic glial cells	*In vivo*	GITksvNa.7	Retrovirus	2	NA	1MR	Increased local edema
Link CJ Human Gene Res. Inst. Des Moines	HSV-TK treatment of refractory or recurrent ovarian cancer	Ovarian carcinoma cells	*In vivo*	LTKOSN	Retrovirus	0	NA	NA	NA
Mariani L Neuro. Klinik Inselspital Bern	Gene therapy for glioblastoma with HSV-TK	Glioblastoma	*In vivo*	GITK1svNa.7	Retrovirus	6	NA	NA	None

(Continued)

Table III: Continued.

Principal investigator	Protocol title	Target	Gene transduction	Vector name	Delivery vehicle	No. of patients	Evidence of gene expression	No. and type of response	Adverse reaction
Mulder N Acad. Ziekenhuis Groningen	Gene therapy for glioblastoma with HSV-TK	Glioblastoma	*In vivo*	GITK1svNa.7	Retrovirus	3	No	1MR	Seizures, abducens paresis, confusion

Modified from Roth and Cristiano, ref. 68.

be addressed before we can realistically consider GPAT as an effective cancer treatment. At present – as with the majority of gene therapy strategies – GPAT requires efficient delivery systems. Even under optimal preclinical conditions the majority of cells remain untransduced. Similarly, current retroviral systems integrate transgenes randomly within the host genome, and as a consequence, varying patterns of suicide gene expression result, which in turn affects the efficacy of prodrug conversion. For example, studies have demonstrated that tissue-specific expression can often be lost due to promoter interference; as a consequence, improvements in promoter specificty and strength, together with the design of retroviral and adenoviral vectors, must be considered.

In recent years there have been significant advances in our ability to modify both adenoviral and retroviral tropism. However, improvements in the accuracy of the vector often compromise its efficacy. This is particularly true for recombinant retroviruses which provide low titers and more worrisome, poor transduction efficiencies. Such problems could be alleviated by using VPCs to maintain a high titer of virus around the tumor site. Alternatively, it may be necessary to consider the development of synthetic vectors. This, together with the design of chimeric transcriptional elements (to increase tumor specificity and improve transgene expression) coupled with the potential to design "tailor-made" prodrugs and chimeric enzymes makes the future for GPAT look promising.

References

1. Roth, J.A., Muthopadhyay, T., Zhang, W.W., Fujiwara, T., Georges, R. *Gene replacement strategies for the prevention and treatment of cancer.* Eur J Cancer 1994, 30A: 2032-7.

2. Culver, K.W., Blaese, R.M. *Gene therapy for cancer*. Trends Genet 1994, 10 (5): 174-8.

3. Mullen, C.A. *Metabolic suicide genes in gene therapy.* Pharmacol Ther 1994, 63: 199-207.

4. Chen, L., Waxman, D.J. *Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P-450 gene transfer: Development of a combined chemotherapy/cancer gene therapy stratergy*. Cancer Res 1995, 55: 581-9.

5. Connors, T.A. *The choice of prodrugs for gene directed enzyme prodrug therapy of cancer*. Gene Ther 1995, 2: 702-9.

6. Hirsch, M.S., Kaplan, J.C. *Antiviral agents.* In: Virology, 2nd Ed. Fields, B.N. et al. (Eds.). Raven Press: New York 1990, 441-68.

7. Miller, W.H., Miller, R.L. *Phosphorylation of acyclovir (acycloguanosine) monophosphate by GMP kinase*. J Biol Chem 1980, 255: 7204-7.

8. Reid, R., Mar, E.C., Huang, E.S., Topal, M.D. *Insertion and extension of acyclic, dideoxy and ara nucleotides by herpes viridae, human alpha and human β polymerasees. A unique inhibition mechanism for 9-(1,3-dihydroxy-2-propoxymethyl) guanine triphosphate.* J Biol Chem 1988, 263: 3898-904.

9. Mar, E.C., Chiou, J.F., Cheng, Y.C., Huang, E.S. *Inhibition of cellular DNA polymerase α and human cytomegalovirus-induced DNA polymerase by triphosphates of 9-(2-hydroxyethoxymethyl) guanine and 9-(1,3-dihydroxy-2-propoxymethyl) guanine*. J Virol 1985, 53: 776-80.

10. Wallace, H. et al. *Specific ablation of thyroid follicle cells in adult transgenic mice*. Endocrinology 1991, 129: 3217-26.

11. Allen, R.G. et al. *Targeted abalation of pituitary pre-proopiomelanocortin cells by herpes simplex virus-1 thymidine kinase differentially regulates mRNAs encoding adrenocorticotropin receptor and aldosterone synthase in the mouse adrenal gland.* Mol Endocrinol 1995, 9: 1005-16.

12. Cranfield, V., West, B., Goldenring, I.R., Levenson, R. *Genetic abalation of parietal cells in transgenic mice: A new model for analysing cell lineage relationships in the gastric mucosa.* Proc Natl Acad Sci USA 1996, 93: 2431-5.

13. Wallace, H. et al. *Ganciclovir-induced ablation of non-proliferating thyrocytes expressing herpes virus thymidine kinase occurs by p53 independent apoptosis*. Oncogene 1996, 13: 61.

14. Bi, W.L., Parysek, L.M., Warnick, R., Stambrook, P.J. *In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSVtk retroviral gene therapy*. Hum Gene Ther 1993, 4: 725-32.

15. Vile, R.G., Nelson, J.A., Castleden, S., Chong, H., Hart, I.R. *Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component.* Cancer Res 1994, 54: 6223-34.

16. Freeman, S., Abboud, C.N., Whartenby, K.A. et al. *The "bystander effect": Tumor regression when a fraction of the tumor mass is genetically modified.* Cancer Res 1993, 53: 5274-83.

17. Averett, D.R., Koszalka, G.W., Fyfe, J.A., Roberts, G.B., Purifoy, D.J.M., Krenitsky, T.A. *6-Methoxypurine arabinoside as a selective and potent inhibitor of varicella-zoster virus. Antimicrob Agents Chemother 1991, 35: 851-7.*

18. Danielsen, S., Kilstrup, M., Barilla, K., Jochimsen, B., Neuhard, J. *Characterization of the Escherichia coli codBA operon encoding cytosine permease and cytosine deaminase*. Mol Microbiol 1992, 6: 1335-44.

19. Harris, J.D., Gutierrez, A.A., Hurst, H.C., Sikora, K., Lemoine, N.R. *Gene therapy for cancer using tumour-specific prodrug activation.* Gene Ther 1994, 1: 170-5.

20. Huber, B.E., Austin, E.A., Good, S.S., Knick, V.C., Tibbels, S., Richards, C.A. *In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase*. Cancer Res 1993, 53: 4619-26.

21. Knox, R.J., Friedlos, F., Boland, M.P. *The bioactivation of CB 1954 and its use as a prodrug in antibody-directed enzyme prodrug therapy (ADEPT).* Cancer Metastasis Rev 1993, 12: 195-212.

22. Clark, A.J., Iwobi, M., Cui, W. et al. *Selective cell ablation in transgenic mice expressing E. coli nitroreductase.* Gen Ther 1997, 4: 101-10.

23. Wei, M.X., Tamiya, T., Rhee, R.J., Breakefield, X.O., Chiocca, E.A. *Diffusible cytotoxic metabolites contribute to the in vitro bystander effect associated with cyclophosphamide/ cytochrome P450 2B1 cancer gene therapy paradigm*. Clin Cancer Res 1995, 1: 117-7.

24. Moolten, F.L. *Tumour chemosensitivity conferred by inserted herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy*. Cancer Res 1986, 46: 5276-81.

25. Miller, N., Vile, R. *Targeted vectors for gene therapy*. FASEB J 1995, 9: 190-9.

26. Garver, R.I., Goldsmith, K.T., Rodu, B., Hu, P.-C., Sorscher, E.J., Curiel, D.T. *Strategy for achieving selective killing of carcinomas*. Gene Ther 1994, 1: 46-50.

27. Culver, K.W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E.H., Blaese, R.M. *In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors.* Science 1992, 256: 1550-2.

28. Culver, K.W., Vickers, T.M., Lamsam, J.L., Walling, H.W., Seregina, T. *Gene therapy for solid tumours.* Br Med Bull 1995, 51: 192-204.

29. Caruso, M., Panis, Y., Gagandeep, S. et al. *Regression of established macroscopic liver metastases after in situ transduction of a suicide gene*. Proc Natl Acad Sci USA 1993, 90: 7024-8.

30. Oldfield, E.H., Ram, Z., Culver, K.W., Blaese, R.M., DeVroom, H.L., Anderson, W.F. *Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir*. Hum Gene Ther 1993, 4: 39-69.

31. Klatzmann, D., Philippon, J., Valery, C.A., Bensimon, G., Salzmann, J.-L. *Clinical protocol. Gene therapy for glioblastoma in adult patients: Safety and efficacy evaluation of an in situ injection of recombinant retroviruses producing cells carrying the thymidine kinase gene of the herpes simplex type 1 virus, to be followed with the administration of ganciclovir.* Hum Gene Ther 1996, 7: 109-26.

32. Hirschowitz, E.A., Ohwada, A., Pascal, W.R., Russi, T.J., Crystal, R.G. *In vivo adenovirus-mediated gene transfer of the Escherichia coli cytosine deaminase gene to human colon carcinoma-derived tumours induces chemosensitivity to 5-fluorocytosine*. Hum Gene Ther 1995, 6: 1055-63.

33. Hwang, C.H., Smythe, W.R., Elshami, A.S. et al. *Gene therapy using adenovirus carrying the herpes simplex-thymidine kinase gene to treat in vivo models of human malignant mesothelioma and lung cancer.* Am J Respir Cell Mol Biol 1995, 13: 7-16.

34. Smythe, W.R., Hwang, H.C., Amin, K.M. et al. *Recombinat adenovirus transfer of the HSV-thymidine kinase gene to thoracic neoplasma: An effective in vitro drug sensitization system.* Cancer Res 1994, 54: 2055-9.

35. Schnierle, B.S., Groner, B. *Retroviral targeted delivery*. Gene Ther 1996, 3: 1069-73.

36. Russell, S.J., Hawkins, R.E., Winter, G. *Retroviral vectors displaying functional antibody fragments.* Nucleic Acids Res 1993, 21: 1081-5.

37. Chu, T.-M.T., Martinez, I., Sheay, W.C., Dornburg, R. *Cell targeting with retroviral vector particles containing antibody-envelope fusions.* Gene Ther 1994, 1: 292-9.

38. Valsesia-Wittmann, S., Drynda, A., Deleage, G. et al. *Modifications in the binding domain of avian retrovirus envelope protein to redirect the host range of retroviral vectors*. J Virol 1994, 68: 4609-19.

39. Cosset, F.L., Morling, F.J., Takeuchi, Y., Weiss, R.A., Collins, M.K.L., Russell, S.J. *Retroviral retargeting by envelopes expressing an N-terminal binding domain.* J Virol 1995, 69: 6314-22.

40. Valsesia-Wittmann, S., Morling, F.J., Nilson, B.H.K., Takouchi, Y., Russell, S.J., Cosset, F.L. *Improvement of retroviral retargeting by using amino acid spacers between an additional binding domain and the N-terminus of moloney murine leukemia virus SU*. J Virol 1996, 70: 2059-64.

41. Nilson, B.H.K., Morling, F.S., Cosset, F.L., Russel, S.J. *Targeting of retroviral vectors through protease-substrate interactions*. Gene Ther 1996, 3: 280-6.

42. Michael, S.I., Hong, J.S., Curiel, D.T., Engler, J.A. *Addition of a short peptide ligand to the adenovirus fibre protein*. Gene Ther 1995, 2: 660-8.

43. Wickham, T.J., Carrion, M.E., Kovesdi, I. *Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs.* Gene Ther 1995, 2: 750-6.

44. Huber, B.E., Richards, C.A., Krenitsky, T.A. *Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy.* Proc Natl Acad Sci USA 1991, 88: 8039-43.

45. Richards, C.A., Huber, B.E. *Generation of a transgenic model for retrovirus-mediated gene therapy for hepatocellular carcinoma is thwarted by lack of transgene expression.* Hum Gene Ther 1993, 4: 143-50.

46. Kluppel, M., Beermann, F., Puppert, S., Schmid, E., Hummler, E., Schutz, G. *The mouse tyrosinase promoter is sufficient for expression in melanocytes and in the pigmented epithelium of the retina.* Proc Natl Acad Sci USA 1991, 88: 3777-81.

47. Vile, R.G., Hart, I.R. *In vitro and in vivo targeting of gene expression to melanoma cells*. Cancer Res 1993, 53: 962-7.

48. Vile, R.G., Hart, I.R. *Use of tissue specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct injection of DNA*. Cancer Res 1993, 53: 3860-4.

49. Gullick, W.J. *Prevalence of abberrant expression of the epidermal growth factor receptor in human cancers*. Br Med Bull 1991, 47: 87-98.

50. Hollywood, D.P., Hurst, H. *A novel transcription factor, OB21, is required for overexpression of the proto-oncogene c-erbB-2 in mammary tumour lines*. EMBO J 1993, 12: 2369-75.

51. Bosher, J.M., Williams, T., Hurst, H.C. *The developmentally regulated transcription factor AP-2 is involved in c-erbB-2 overexpression in human mammary carcinoma*. Proc Natl Acad Sci USA 1995, 92: 744-7.

52. Richards, C.A., Wolberg, A.S., Huber, B.E. *The transcriptional control region of the human carcinoembryonic antigen gene: DNA sequence and homology studies*. DNA Seq 1993, 4: 185-96.

53. Richards, C.A., Austin, E.A., Huber, B.E. *Transcriptional regulatory sequences of carcinoembryonic antigen: Identification and use with cytosine deaminase for tumorspecific gene therapy.* Hum Gene Ther 1995, 6: 881-93.

54. Schrewe, H., Thompson, J., Bona, M. et al. *Cloning of the complete gene for carcinoembryonic antigen: Analysis of its promoter indicates a region conveying cell-type specific expression.* Mol Cell Biol 1990, 10: 2738-48.

55. Jothy, S., Yuan, S.Y., Shirota, K. *Transcription of carcinoembryonic antigen in normal colon and colon carcinoma. In situ hybridization study and implication for a new in vivo functional model.* Am J Pathol 1993, 143: 250-7.

56. Osaki, T., Tanio, Y., Tachibana, I. et al. *Gene therapy for carcinoembryonic antigen-producing human lung cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene.* Cancer Res 1995, 54: 5258-61.

57. Fujita, K., Sugaya, S., Ishii, S., Tanaka, K. *Specific expression of Pseudomonas exotoxin in tumour cells promoted by myc binding sequence.* Cancer Gene Ther 1993/1994, 1 (Suppl. 1): 3.

58. Kumagai, T., Tanio, Y., Osaki, T. et al. *Eradication of myc-overexpressing small lung cancer cells transfected with herpes simplex virus thymidine kinase gene containing myc-max response elements.* Cancer Res 1996, 56: 354-8.

59. Su, H., Chang, J.C., Xu, S.M., Kan, Y.W. *Selective killing of AFP-positive hepatocellular carcinoma cells by adeno-associated virus transfer of the herpes simplex virus thymidine kinase gene*. Hum Gen Ther 1996, 7: 463-70.

60. Siders, W.M., Halloran, P.J., Fenton, R.G. *Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells*. Cancer Res 1996, 56: 5638-46.

61. Ring, C.J.A., Harris, J.D., Hurst, H.C., Lemoine, N.R. *Suicide gene expression induced in tumour cells transduced with recombinant adenoviral, retroviral and plasmid vectors containing the ERBB2 promoter.* Gene Ther 1996, 3: 1094-103.

62. Vile, R., Miller, N., Chernajovsky, Y., Hart, I. *A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes.* Gene Ther 1994, 1: 307-16.

63. Vile, R.G., Diaz, R.M., Miller, N., Mitchell, S., Russell, S.J. *Tissue-specific gene expression from Mo-MLV retroviral vectors with hybrid LTRs containing the murine tyrosinase enhancer/promoter*. Virology 1995, 214: 307-13.

64. Mullen, C.A., Coale, M.M., Lowe, R., Blaese, R.M. *Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor.* Cancer Res 1994, 54: 1503-6.

65. Freeman, S., Whartenby, K., Freeman, J., Abboud, C., Marrogi, A. *In-situ use of suicide genes for cancer therapy. Sem* Oncol 1996, 23: 31-45.

66. Ramesh, R., Marrogi, A., Munshi, A., Abboud, C., Freeman, S. *In vivo analysis of the bystander effect: A cytokine cascade.* Exp Haematol 1996, 24.

67. Santodonato, L., Ferrantini, M., Gabriele, L. *Cure of mice with established metastatic Friend leukaemia cell tumours by a combined therapy with tumour cells expressing both interferon-α_1 and herpes thymidine kinase followed by ganciclovir.* Hum Gene Ther 1996, 7: 1-10.

68. Roth, J.A., Cristiano, R.J. *Gene therapy for cancer: What have we done and where are we going?* J Nat Cancer Ins 1997, 89 (1): 21-37.

69. Eck, S.L., Alavi, J.B. *Treatment of advanced CNS malignancy with the recombinant adenovirus H5.020RSVTK:A phase I trial*. Hum Gene Ther 1995, 6(4): 509-12.

70. Albelda, S.M. *Treatment of advanced mesothelioma with recombinant adenovirus H5.020RSVTK: A phase I trial.* Hum Gene Ther 1995, 6(4): 509-14.

71. Grossman, R.G., Woo, S.L.C. *Phase I study of adenoviral vector delivery of the HSV-TK gene and the intravenous administration of ganciclovir in adults with malignant tumors of the central nervous system*. Hum Gene Ther 1995, 6(8): 1090-3.