REVIEWS

Gene-Directed Enzyme Prodrug Therapy

Ion Niculescu-Duvaz, Robert Spooner, Richard Marais,† and Caroline J. Springer*

CRC Centre for Cancer Therapeutics at the Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, United Kingdom, and CRC Centre for Cells Molecular Biology at the Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, United Kingdom. Received June 21, 1997

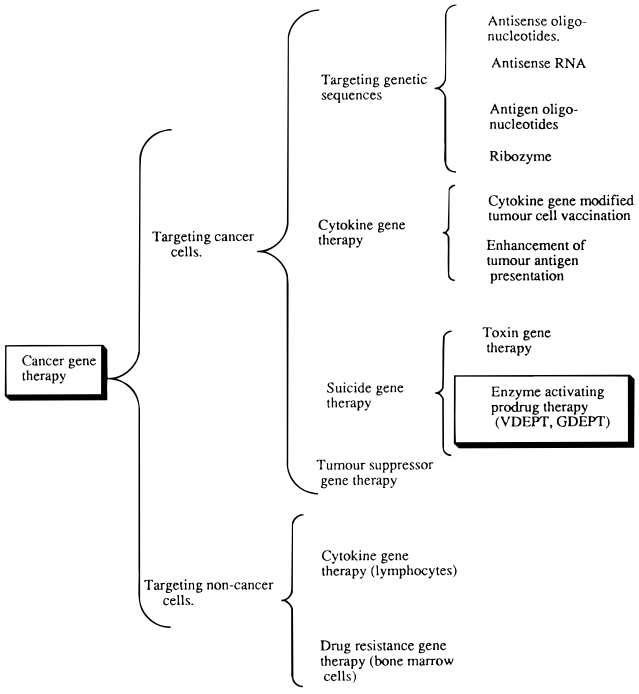
TABLE OF CONTENTS

1. INTRODUCTION

Gene therapy may be broadly defined as a genetic technology aimed at modifying cells for therapeutic gain. There have been many proposals to modify genetically, or to suppress gene expression in, tumor cells (*1*). An early suggestion was antisense oligonucleotide strategies that target specific mRNA sequences. Another approach is the "antigene oligonucleotide" approach, which aims to target DNA sequences. Other strategies involve antisense RNA (which interferes with transcription and translation, blocks RNA splicing, or induces double-stranded DNA cleavage) and antioncogene ribozymes (*1*, *2*). Cytokine gene therapy has also been proposed in an attempt to elicit an immune response against tumor cells and thus enhance the patient's immune system to destroy the tumor. The introduction of tumor suppressor genes is also being investigated (*1*).

Other approaches include attempts to stabilize normal tissues, especially those that are rapidly dividing (e.g., bone marrow, duodenum) by expressing enzymes that regulate DNA repair in these tissues but not in the tumor cells. In this approach, it is hoped that the normal tissues which are most sensitive to chemotherapeutic agents can be modified to overcome drug toxicity and thus reduce the side effects of chemotherapy (*3*−*6*). Alternatively, cancer cells may be rendered more sensitive to chemotherapy or toxins, either by suppressing the expression of resistance genes [e.g., multidrug resistance (MDR)] or by introducing "suicide genes", which increase sensitivity to chemotherapeutics (*1*). Hence, it can be seen that it is possible to modify genetically either the malignant or nonmalignant cells for therapeutic gain. There are two suicide gene approaches. The first is a toxin gene therapy, whereby genes are introduced into malignant cells and the protein produced directs the killing of the tumor cells. The second is termed gene-directed enzyme prodrug therapy (GDEPT) (*7*, *8*) or virally directed enzyme prodrug therapy (VDEPT) (*9*). This review will discuss the GDEPT system of making tumor cells more sensitive to chemotherapeutic agents. (See Scheme 1.)

Scheme 1. Cancer Gene Therapy



In GDEPT (or VDEPT), foreign enzymes are expressed in target cells, where they can activate subsequently administered nontoxic prodrugs in to active drugs.

To achieve success in GDEPT, a number of factors must be accomplished. GDEPT requires (1) a gene expressing a nonendogenous enzyme or an endogenous enzyme that is poorly expressed in tumors, which is able to activate a prodrug; (2) a means of delivering the gene to the target cells; and (3) a prodrug.

The gene must be expressed exclusively or with a relatively high ratio in tumor cells compared to normal tissues and blood. The catalytic activity of the foreign or endogenous enzymes, which are only expressed at low concentrations, should be high enough to achieve therapeutic benefit, which may require a specific threshold to be reached. A wide range of enzyme/prodrug systems have been considered. (See section 3.)

The gene should be expressed exclusively or with relatively high ratio in tumor cells compared to normal tissues and blood and must occur in sufficient number

* Author to whom correspondence should be addressed (telephone 44-181-643 8901; fax 44-181-770 7899).
† CRC Centre for Cells Molecular Biology at the Institute of Cancer Research.

S1043-1802(97)00116-X CCC: $15.00 © 1998 American Chemical Society
Published on Web 01/12/1998

of cells that there be a clinical benefit (see section 4 for bystander effect). After gene delivery, prodrug administration must be delayed to permit protein expression and stable accumulation in the targeted cells, and the catalytic activity of the protein should be high enough to achieve a therapeutic benefit at these expression levels, which may require a specific threshold to be reached.

2. VECTORS USED IN GDEPT

A number of vector systems have been proposed for gene therapy. These include the RNA viruses: retroviruses (*10*); the DNA viruses: adenovirus, parvovirus (*10*, *11*), and herpes simplex virus (*12*); cationic lipids (*13*, *14*), peptides (*15*), and naked DNA (*16*). In addition, antibody-directed and ligand-directed (*17*) delivery of DNA have been proposed. There has also been a suggestion that the anaerobic bacterium *Clostridium* could be used for targeting to the hypoxic regions of tumors (*18*).

The majority of the proposals have been for viral vectors that have been modified to be deficient in replication functions, although the use of replication-competent viruses has also been considered (*19*). For applications such as *ex vivo* infections, direct administration of vectors to target tissues *in vivo* or for locoregional delivery, the ability to target malignant cells specifically may not be necessary. However, if systemic delivery is used, targeting to malignant cells is an absolute necessity. Some viruses have restricted tropisms that may be of value. For example, the minute virus of mice is oncotropic and can deliver DNA to transformed cells (*20*); similarly, the human parvovirus B19 targets primary hematopoietic progenitor cells. The B19 tropism can be transferred to adeno-associated virus by recombinant means (*21*). The two groups of viruses that have been most extensively studied, retroviruses and adenoviruses, have much less restricted tropisms, and efficient targeting in the clinic is, as yet, unaccomplished. The manipulation of these viruses to modify their tropism by altering their coat proteins is described below. Furthermore, the possibility of ensuring expression of therapeutic genes in particular subsets of cells, by modifying the regions that control gene expression and by adding promoter sequences that will only be active in malignant cells, is discussed.

The issue of transfer efficiency is of paramount importance in GDEPT. *Ex vivo* transfer efficiencies of ~100% have been achieved with viral carriers. However, *in vivo* transfer efficiencies are much lower, and conflicting data are reported.

Retroviruses mediate stable gene transfer with low potential immunogenicity. Difficulties in producing high-titer virus (maximum 10^7 cfu mL^{-1}), the fact that only dividing cells are infected, and the possibility of insertional mutations are nevertheless serious drawbacks. Direct injection of retrovirus in brain tumors resulted in low efficiency (1−3%) (*22*). However, 10−55% transfer efficiencies were reported in the case of direct implantation of retrovirus producer cells in such systems (*22*).

Adenoviruses as alternative vectors, have the advantage of producing higher titers (maximum 10^{11}−10^{12} pfu mL^{-1}) capable of generating infections regardless of the proliferative status of the cells (*23*). Efficiency ≃50% (at virus titer, 10^{10} pfu mL^{-1}) was observed in mouse hepatocytes after an intravenous injection of adenovirus carrying the lac Z gene (*24*).

2.1. Retroviruses. Most of the retroviral vectors currently being examined for gene therapy have been derived from the Moloney murine leukaemia virus (MoMuLV). There are of two types, defined by their infection spectrum: ecotropic, which infect murine cells only; or amphotropic, which can infect a wide range of mammalian cells, including human and murine cells. Neither ecotropic nor amphotropic viruses display tissue specificity, although different cell types within the host organism differ in their sensitivity to infection and some cells are very resistant (e.g., hepatocytes and early hematopoietic progenitor cells). Therefore, for targeting specific populations of cells *in vivo*, a means of altering retrovirus specificity would be of great value.

Mammalian type C retroviruses can infect human cells *in vitro*, but since they are inactivated by human serum, transmission of these viruses to humans is restricted. Inactivation correlates in part with the sensitivity of producer cells to lysis by human serum, so that, for example, retroviruses produced from murine or dog cells are all sensitive to human serum while viral particles from human cells are more resistant (*25*). Exposure of sensitive producer cells and sensitive retrovirus to human serum would presumably decrease gene transfer *in vivo*, a serious barrier for gene therapy. It had been thought that antibody-independent activation of the classical complement pathway was responsible, due to direct binding of human C1q (the first component of this pathway) to retroviral envelope proteins. However, recently, this view has been challenged, since anti-α-galactosyl antibodies in human serum inactivate retroviruses whose envelopes contain the Galα1−3Galβ1−4GlcNAc-R carbohydrate epitope (*26*, *27*). These experiments have implications for the production of retroviruses for gene therapy: soluble Galα1−3Gal can protect retrovirus from human serum and might be administered with the retrovirus. Alternatively, production of retrovirus from producer cell lines that do not express α-galactosyl modifications should permit production of retroviral particles with prolonged survival in human serum.

Another recent advance has been the discovery of a number of packaging cell lines that produce high-titer recombinant retroviruses, and these have been engineered to be resistant to human serum (*28*, *29*). These cell lines will overcome the technical barriers that prevent production of the large number of viral particles required for systemic administration of retrovirus into patients. It should be remembered that, although human serum inactivates many retroviruses, both retroviruses and producer cells survive exposure to cerebrospinal fluid and fluid from the tumor bed in patients with malignant glioma (*30*, *31*). Thus, gene transfer from nonnmodified retroviruses to tissues within the central nervous system may be more successful than gene transfer to other tissues.

Retroviral host range is determined by the interaction of the retrovirus envelope (env) glycoprotein with host cell surface proteins (*32*). Glycoprotein env is synthesised as a precursor, which is proteolytically cleaved into two disulfide-linked proteins. In MoMuLV, one is the hydrophobic p15(E) transmembrane protein (TM) responsible for tethering env to the viral particle, and the other is the hydrophilic glycosylated surface gp70 protein that is responsible for interacting with host cell receptors.

These env host cell receptor interactions can be overcome by biochemical means to retarget virus particles. Bispecific antibody complexes have been prepared by using streptavidin to link biotinylated anti-major histocompatibility complexes (MHC) class I antibodies with biotinylated anti-ecotropic receptor antibodies and to link biotinylated anti-MHC class II antibodies with biotinylated anti-ecotropic receptor antibodies. These complexes

can act as bridges permitting infection of human cells (*33*). Similar bispecific complexes have permitted targeting via the epidermal growth factor receptor and the insulin receptor, but this system is not universal, since it does not work via the transferrin, high-density lipoprotein, or galactose receptors (*34*). However, chemical conjugation of lactose to ecotropic virus particles allows infection of human hepatic cells, presumably via the galactose receptor (*35*).

Some proteins, such as Thy-1 (*36*), CD4 (*37*), β2-microglobulin, HLA DR, actin, and ubiquitin (*38*), can be incorporated naturally into retroviral particles and so have the potential to influence retroviral tropism. Many approaches have followed a more directed route, specifically altering env to attempt retroviral retargeting. Since structural variations of gp70 determine whether the virus has ecotropic or amphotropic host range, one approach to targeting might be to engineer the *env* gene to confer new tissue specificity to the env protein. Early attempts fused DNA encoding a single-chain Fv antibody fragment (scFv) specific for a hapten with DNA encoding the env glycoprotein of MoMuLV (*39*) and spleen necrosis virus (*40*). When coexpressed with a retrovirus expressing wild-type env, retrovirus particles that could bind hapten were recovered. The hapten specificity had been overlaid upon the normal cell tropism, but the presence of wild-type env meant that normal tropism was not ablated.

Similar approaches fused a scFv specific for the low-density lipoprotein receptor to MoMuLV env (*41*) and a scFv specific for a cell-surface antigen expressed on human colon carcinoma cells to spleen necrosis virus env (*42*). Other targeting motifs fused to env include a 16 amino acid peptide containing the integrin-binding motif RGD fused to avian leukosis virus env (*43*); erythropoietin fused to MoMuLV env (*44*); heregulin, a ligand for human epidermal growth factor receptors 2 and 4, fused to MoMuLV env (*45*); CD4, for targeting HIV-infected cells fused to MoMuLV env (*46*); epidermal growth factor (*47*); and the first 208 amino acids of amphotropic virus env fused to murine leukemia virus ecotropic env (*47*). In all cases, expression was in packaging cell lines that also expressed the appropriate wild-type env, resulting in viral particles with an overlaid specificity that permitting redirected cell binding, although not always productive infection (for example, the EGF-env fusion was blocked at a postbinding stage) (*47*).

A substantial reduction in postbinding block and up to a 100-fold increase in gene transfer efficiency were achieved by introducing spacer peptides separating the amphotropic and ecotropic moieties of an ampho/ecotropic fusion env (*48*). If this proves to be a general phenomenon, it might be of great value for gene therapy.

The requirement for wild-type env glycoprotein has led to the idea that it may act as a helper function (*42*), but it may depend upon the nature of the fused ligand. A scFv (anti-MHC class I)-ecotropic env fusion permitted retroviral binding to, and infection of, human cells, even when produced from cells that did not coexpress wild-type env (*49*). A strategy that may also target cells efficiently relies upon the fusion of the targeting ligand to env via a protease-sensitive linker (*50*). Here, cell-specific targeting was determined by the fused EGF ligand, but the ligand blocked the env−receptor interaction and gene delivery did not proceed until the linker was cleaved.

Despite the generally low efficiencies of gene transfer, the principle of retargeted retroviruses is now well established, although the criticism that the important event may simply be to concentrate the modified retrovirus to a particular cell surface cannot be ignored (*51*).

2.2. Adenoviruses. The adenoviral capsid is icosahedral, each vertex terminating in a penton base structure that interacts with cellular integrins. A trimeric fiber that terminates in a trimeric knob is inserted into each penton base. The knob is the primary ligand for the cellular adenoviral receptor. Serotypes differ, in part, in the length of the fiber shaft. A chimeric fiber, with the shaft of adenovirus serotype 5 (Ad5) fused to the knob of Ad3, confers Ad3-type specificity (*52*). Thus it is possible to alter adenoviral specificity by manipulating the knob domain. A refinement was the introduction of a decapeptide encoding part of the gastrin releasing peptide (GRP) to the carboxy terminus (knob) of an adenoviral fiber (*53*). The resulting fiber could still trimerize, and the peptide was accessible to an anti-GRP antibody, raising hopes that incorporation of such a fiber into an adenovirus might permit targeting to cells expressing GRP receptors. Whether such manipulations ablate normal targeting is not clear, although it is known that there are considerable constraints in knob structure. While the shaft can tolerate large deletions, removal of just the two carboxy-terminal amino acids of the Ad2 knob results in monomeric fibers, as does addition of a 27-mer peptide (*54*). To date, none of these targeting strategies has been attempted for a GDEPT approach to cancer.

2.3. Transcriptional Targeting. Another area in which malignant cell targeting progress has been made is at the transcriptional level. If a tumor cell overexpresses a particular protein due to increased specific transcriptional activity of its promoter (rather than through gene duplication), this characteristic can be exploited for the GDEPT approach. Foreign genes can be cloned downstream of the promoter and introduced into appropriate tumor cells to allow specific high-level expression of the protein. Here, normal tissue that is also transduced would express much lower levels of the gene product and in an ideal system, none.

High-level expression of hepatoma-associated α-fetoprotein (AFP) makes hepatocellular carcinoma (HCC) a prime target for this approach (*55*). Varicella zoster virus (VSV) thymidine kinase (TK) was cloned under the transcriptional control of specific promoters, the AFP tissue regulatory sequence (TRS) and the albumin (ALB) TRS, in replication defective retroviruses. Infection of Hep G2 hepatoma cells and HuH7 non-hepatoma cells, both of which express AFP and ALB, resulted in greatly increased sensitivity to the prodrug 6-methoxypurine arabinonucleoside (ara-M) (a good substrate for VZV-TK, see section 3.1). If these systems were used *in vivo*, further levels of selectivity might be expected, due to failure of retrovirus to infect nondividing hepatocytes and the lack of toxicity of ara-ATP to nondividing cells. A retrovirus expressing HSV-TK under AFP regulation control conferred ganciclovir (GCV) sensitivity to infected AFP-producing cells, but not to cells that did not express AFP (*56*). In addition, the presence of a glucocorticoid-responsive element in the AFP promoter permitted enhancement of acyclovir (ACV)-activated cytotoxicity by administration of dexamethasone, raising the possibility of pharmacological modulation of therapy.

Similarly, replication defective adenovirus expressing herpes simplex virus HSV-TK from AFP promoter/enhancer elements increased sensitivity of Hep G2 and HuH7 cells to GCV, but did not increase sensitivity of cells that did not express AFP (*24*). In murine tumor models, gene transfer by adenovirus delivery of HSV-TK

under AFP promoter/enhancer control and subsequent GCV administration led to inhibition of HCC growth (*57*). In nude mice, adenoviral transduction of HSV-TK expressed from a Rous sarcoma virus promoter led to nonspecific killing of human tumor cells upon subsequent administration of GCV, but using the AFP promoter restricted cell death to only those cells expressing AFP, leading to complete regression of HuH7 tumors (*58*). In transgenic mice, expression of HSV-TK was found to be predominantly in the liver when under the transcriptional control of an AFP/enhancer/ALB promoter fusion, demonstrating the high degree of tissue specificity of this promoter system (*59*). Adenovirus-associated virus delivery of this construct followed by GCV treatment was shown to kill cells expressing both AFP and ALB, but not cells expressing neither.

A range of tissue-specific promoters has been suggested for use in the GDEPT approach. Secretory leukoprotease inhibitor is expressed in a wide range of carcinomas, including lung, breast, ovarian, bladder, oropharyngeal, and colorectal, and its promoter has been used to express HSV-TK (*60*). Osteocalcin is a noncollagenous bone matrix protein expressed at high level in osteoblasts. Its promoter has also been used to express HSV-TK and, when delivered to cells in an adenovirus vector, results in TK activity in both murine and human osteoblast cell lines. There was no expression in a panel of murine and human nonosteoblast cell lines, and subsequent treatment with ACV led to cell death only in the targeted cells (*61*). Intratumoral (i.t.) injection of this adenovirus into murine osteosarcoma followed by intraperitoneal (ip) injection of ACV abolished tumor growth. For small-cell lung cancers that overexpress Myc, transcriptional control of HSV-TK by the Myc-Max response elements appears to be of value (*62*).

Carcinoembryonic antigen (CEA) is overexpressed by a number of carcinomas. Transfection of HSV-TK under the transcriptional control of a truncated (445 bp) CEA promoter into a CEA-producing human lung cancer cell line increased its sensitivity to GCV, while transfection into two CEA-nonproducing lines, a lung cancer cell line and HeLa cells, did not. In nude mice, significant regressions of CEA-producing lung cancer tumors expressing this CEA/HSV-TK construct were obtained after GCV treatment (*63*). Tumors were derived by subcutaneous (sc) implantation of CEA-expressing pancreatic carcinoma cells transduced with a retrovirus expressing HSV-TK downstream from a CEA promoter into severe combined immunodeficient (SCID) mice (*64*). After GCV treatment, reductions in tumor size were noted. An adenovirus expressing HSV-TK from the CEA promoter conferred GCV sensitivity to CEA-producing gastric carcinoma cell lines, but not to CEA-nonproducing cell lines or to HeLa cells (*65*). Optimization of expression by recombinant techniques has resulted in a small CEA promoter (important because of virus packaging limitations) that can direct high-level expression of cytosine deaminase (CD) in CEA-positive cells (*66*).

The c-erb B2 proto-oncogene is overexpressed in 25−30% of breast and pancreatic carcinomas, ~40% of non-small-cell lung cancers (NSCLC), 20−30% of ovarian carcinomas, and 20% of stomach and bladder carcinomas. In breast cancer, overexpression correlates with poor prognosis and resistance to chemotherapy, so a new treatment modality is very important. Overexpression of c-erb B2 occurs in part at the level of gene transcription, owing to elevated levels of a developmentally regulated transcription factor (*67, 68*). Expression of CD from a truncated c-erb B2 promoter has been proposed for VDEPT in cells overexpressing c-erb B2 (*69*). After treatment of retrovirally transduced cells with 5-fluorocytosine (5-FC), significant cell death was noted in c-erb B2-positive cell lines but not in c-erb B2-negative cell lines. The enhancer sequence of the DF3/MUCI gene that encodes a polymorphic high molecular weight glycoprotein can also regulate TK expression, limiting it to breast cancer cells (*70*). Similarly, the DF3 promoter has been used in adenovirus to confer selective expression of HSV-TK in DF3 positive breast carcinoma cell lines (*71*).

A problem frequently encountered using retroviral vectors containing tissue-specific promoters is interference with specificity from the viral enhancer in the 3′ long terminal repeat (LTR). For example, the SV40 enhancer and the immunoglobulin heavy chain enhancer both promote gene expression in lymphoid cells. However, introduction of these promoter regions into retroviral vectors that express HSV-TK fails to enhance expression in a murine lymphoma cell line (*72*).

Melanin biosynthesis is restricted to melanocytes due to transcriptional regulation. The tyrosinase promoter has been used successfully for specific expression of genes in melanoma cells (*73*−*75*), as has the promoter for tyrosinase-related protein 1 (*76*). Recently, a truncated (769 bp) tyrosinase promoter/enhancer has been used to replace the enhancer in the 3′ LTR of MoMuLV (*10*). The result was abolition of promoter interference, with very low expression levels of the downstream gene, interleukin 2, in nonmelanoma cell lines, but high-level expression in melanoma cell lines infected with the retrovirus. Increasing the length of the tyrosinase promoter/enhancer to 2.5 kb improved levels of gene expression further. If such an approach can be extended to other tissue-specific or cancer-specific promoters, then powerful tools for transcriptionally regulated gene transfer should become available.

3. ENZYMES/PRODRUG SYSTEMS USED IN GDEPT

Enzymes used in GDEPT should meet some specific requirements. Ideally they should be: able to catalyze scission reactions or other types of reactions (e.g., redox reactions); different from any circulating endogenous enzyme accessible to prodrug; expressed in sufficient amounts in the tumor target cells; and able to effect high catalytic activity. It may be possible to improve on the enzymes that are currently available. For example, mutants have been created in viral TK that improved the kinetics of the enzyme for the prodrugs GCV and ACV (see section 3.2) (*77*). Alternatively, the prodrug may be modified to make it a better substrate for the enzyme to overcome enzyme inadequacies.

The enzymes proposed for GDEPT are summarized in Table 1 and can be characterized into two classes: (a) One class consists of enzymes of nonhuman origin, e.g., viral TK, *Escherichia coli* CD, *Pseudomonas* sp. carboxypeptidase, *E. coli* purine nucleotide phosphorylase, *E. coli* xanthine−guanine phosphoribosyltransferase, and *E. coli* nitroreductase. They may elicit cell-mediated immune responses in humans, which may be an advantage, producing more extensive bystander cytotoxicity (see section 4). In experiments with cytosolically expressed herpes simplex virus thymidine kinase in mouse of malignant melanoma (*78*) and colon carcinoma (*79*), the bystander effect was greater in immunocompetent mice than in nude mice and was diminished after sublethal irradiation, implying a strong immune component. After treatment with GCV, tumor deposits had greater leukocyte infiltration than those of control animals (*80*) and

Table 1. Enzymes Used in GDEPT

no.	enzyme	code	source	reaction catalyzed by the enzyme	comments	refs
1	thymidine kinase	EC 2.7.1.21	herpes simplex virus	phosphorylation of GCV, ACV, and FIAU	exogenous, viral origin	82
	thymidine kinase	EC 2.7.1.21	varicella zoster virus	phosphorylation of ara-M (6-methoxypurine); poor activity against GCV, ACV, and FIAU	exogenous, viral origin	52, 81
2	cytosine deaminase	EC 3.5.4.1	*E. coli*	conversion of cytosine to uracil (or 5FC to 5FU)	exogenous, bacterial origin	106−108
3	cytochrome P450		rat	activation of oxazaphosphorines (CP, IF, etc.) by hydroxylation of the oxazaphosphorine cycle	exogenous, CYP2B1, mammalian origin; its human counterpart, CYP2B6 is expressed in liver but not in tumor cells	116
4	purine nucleoside phosphorylase	EC 2.4.2.1	*E. coli*	reversible phosphorylation of purine nucleosides	exogenous; human counterpart expressed in erythrocytes but presents important differences in specificity	122
5	thymidine phosphorylase	EC 2.4.2.4	human	reversible phosphorolytic cleavage of thymidine, deoxyuridine, and their analogues	endogenous, low level in tumor cells	124
6	deoxycytidine kinase	EC 2.7.1.74	human	phosphorylation of deoxycytidine (but as well some purine deoxynucleosides)	endogenous, low level in tumur cells	125
7	carboxypeptidase G2	EC 3.4.22.12	*Pseudomonas* sp.	cleavage of the amidic bond between an aromatic nucleus and glutamic acid	exogenous, bacterial origin	127
8	xanthine−guanine phosphoribosyl-transferase	EC 2.4.2.22	*E. coli*	catalyzes the conversion of hypoxanthine, xanthine, and guanine to IMP, XMP, and GMP	bacterial origin with human counterpart (HPRT)	135
9	nitroreductase		*E. coli*	reduction of aromatic nitro groups	exogenous, bacterial origin having a human counterpart with different substrate specificity	134

cytokine release from these was implicated in up-regulation of the costimulatory molecules B7 and ICAM and with enhanced host T-cell proliferative response.

(b) The other class of enzymes are of human origin and are not present or are expressed only at low concentrations in tumor cells, e.g., deoxycytidine kinase, thymidine phosphorylase, and cytochrome P450. Their main advantage resides in the reduction of the potential for inducing an immune response. However, their presence in normal tissues is likely to preclude specific activation of the prodrugs only in tumors.

The prodrug/drug system for GDEPT should also meet a number of requirements: the prodrug must be able to cross the tumor cell membrane for intracellular activation; the cytotoxicity differential between the prodrug and its corresponding drug should be as high as possible; the prodrug should be a good substrate for the expressed enzyme but not activated by endogenous enzymes in other tissues; and the active drug should be highly diffusible or be actively taken up by neighboring cells to achieve the bystander effect (see section 4). The differential between the prodrug and the active drug should be as large as possible.

The design of a prodrug with low cytotoxicity which can release highly toxic active drug requires effective knowledge of the structure−activity relationship (SAR) or preferably of the quantitative structure−activity relationship (QSAR) for the particular type of compound. For this reason, most of the prodrugs used in GDEPT to date have been compounds that have already been used clinically as anticancer agents. An additional advantage of this approach is that the pharmacological, pharmacokinetic, dosage, and safety parameters of these compounds are known.

To date there have been many different prodrugs/enzyme combinations that have been tested in various GDEPT systems. They are summarized in Table 2.

The rationale for their use and the results obtained with each of them are discussed below.

3.1. 6-Methoxypurine Arabinonucleoside (Ara-M)/Varicella Zoster Virus Thymidine Kinase (VZV-TK). The rationale for this prodrug choice was that ara-M is a good substrate for VZV-TK but a poor substrate for the three major mammalian nucleotide kinases (*55*, *81*). The prodrug is activated by the viral enzyme to ara-M-monophosphate (ara-MMP), which is then further converted by the mammalian AMP-deaminase to inosine arabinonucleoside monophosphate (ara-IMP). This metabolite is further catalyzed by mammalian adenylosuccinate synthetase lyase into adenine arabinonucleoside monophosphate (ara-AMP), which is then phosphorylated by the AMP-kinase and subsequently by the cellular nucleoside diphosphate kinases to the anabolite adenine arabinonucleoside triphosphate (ara-ATP) (see Scheme 2). An alternate possible pathway is through hypoxanthine arabinonucleoside (ara-H), which involves the adenosine deaminase-catalyzed demethoxylation of ara-M. Ara-ATP exerts its cytotoxic effects on dividing cells, by inhibiting DNA synthesis.

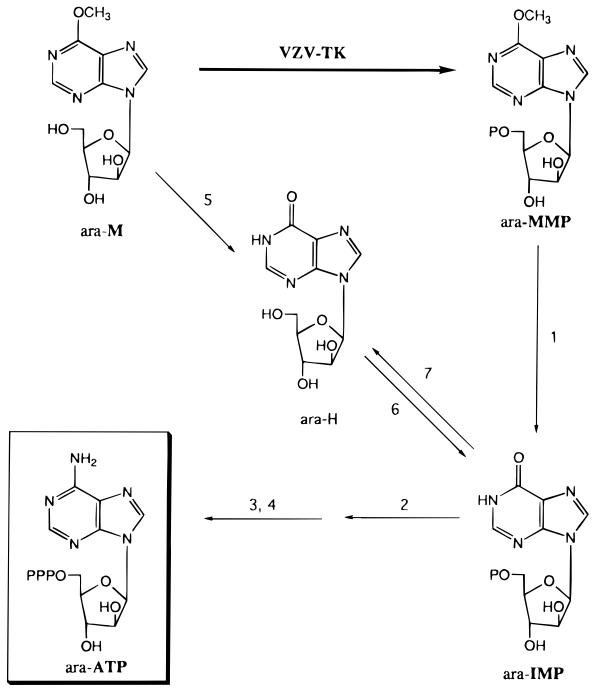
In the absence of VZV-TK, traces of ara-ATP are formed presumably through the intermediate ara-H. Ara-M is converted to ara-H by adenosine deaminase and undergoes further catalysis to ara-IMP by cellular kinases.

Activation of ara-M was used to develop a therapeutic model for the treatment of HCC (*55*) (see also section 2.3). This model takes advantage of the transcriptional differences between normal and malignant cells, by cloning the VZV-TK gene under the transcriptional control of the

Table 2. Prodrugs and Enzymes Used in GDEPT

no.	prodrug	enzyme	refs
1	6-methoxypurine arabinonucleoside	thymidine kinase (varicella zoster virus)	55, 81
2	ganciclovir, acyclovir, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracyl	thymidine kinase (herpes simplex virus)	72−74, 77, 84−105
3	5-fluorocytosine	cytosine deaminase	66, 69, 109−112
4	cyclophosphamide, ifosfamide	cytochrome P450	116−121
5	9-(β-D-2′-deoxy-*erythro*-pentafuranosyl)-6-methylpurine	purine nucleoside phosphorylase	75, 123
6	5′-deoxy-5-fluorouridine	thymidine phosphorylase	124
7	arabinosyl cytosine, 2-chloro-2′-deoxyadenine, 2-fluoro-9-(β-D-arabinofuranosyl)cytosine, 2′,2′-difluorodeoxycytidine	deoxycytidine kinase	125, 126
8	(2-chloroethyl)(2-mesyloxyethyl)aminobenzoyl-L-glutamic acid	carboxypeptidase G2	8
9	5-aziridinyl-2,4-dinitrobenzamide	nitroreductase	7
10	6-thioxanthine, 6-thioguanine	xanthine−guanine phosphoribosyltransferase	136

Scheme 2. Metabolism of Ara-M[a]

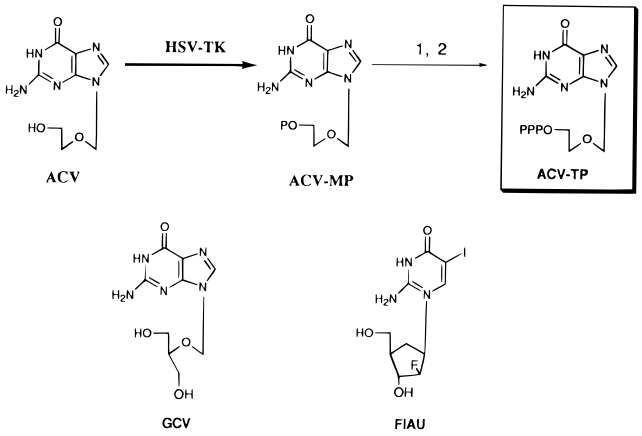


[a] 1, AMP-deaminase; 2, adenylosuccinate synthetase; 3, AMP-kinase; 4, nucleoside diphosphate kinase; 5, adenosine deaminase; 6, cellular kinases; 7, cellular phosphatases.

hepatoma-associated AFP, TRS, or the liver-associated (ALB) TRS. These chimeric tumor and liver specific genes were cloned into replication defective retroviral vectors and used to infect human hepatoma Hep G2 cells (expressing high levels of both ALB and AFP) and HuH7 nonhepatoma cells (expressing high levels of AFP and moderate levels of ALB). In control Hep G2 parental cells, the IC_{50} ratio of prodrug to drug was >2000 (araM, IC_{50} > 2000 μM; araATP < 1 μM). After infection, the IC_{50} of araM was greatly decreased in Hep G2 cells (IC_{50} = 3.1−6.5 μM) as well as in HuH7 cells (IC_{50} = 11−36 μM). The cytotoxicity of ara-M in infected cells paralleled the level of TK activity, and metabolic studies demonstrated an increase in the levels of ara-A of 2000- and 7000-fold in the VZV-TK expressing cells (*55*).

3.2. Purine Nucleosides: ACV, GCV, and 1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracyl (FIAU)/Herpes Simplex Virus TK (HSV-TK). This system is based on the metabolic activation by the HSV-TK of certain purine nucleosides which have previously demonstrated clinical efficacy for the treatment of herpes in humans. HSV-TK catalyzes the phosphorylation of the purine nucleoside analogues ACV, GCV, and FIAU, which are poor substrates for VZV-TK (*81*). The conversion is to the corresponding nucleoside monophosphates, which are catalyzed to nucleoside triphosphates by mammalian nucleoside monophosphate kinases. These latter nucleotides are able to inhibit DNA synthesis and thus kill dividing cells (*82*) (see Scheme 3). Evidence has been obtained for the inhibition of DNA polymerase by ACV-triphosphate, resulting in inhibition of the elongation of newly synthesized DNA. Incorporation of ACV-triphosphate into DNA is also a chain-terminating event, and GCV-triphosphate also acts as a chain terminator (*82*, *83*).

Scheme 3. Metabolism of Acyclovir[a]



[a] 1, human GMP-kinase; 2, nucleoside diphosphate kinases; MP, monophosphate; TP, triphosphate. The same metabolic pathyway occurs for GCV and FIAU.

Cells transfected with the HSV-TK gene and treated with GCV have been shown to die by apoptosis. This process is probably mediated by induction of DNA damage and occurs via a pathway that is independent of p53 (*84*).

The GCV/HSV-TK system is the most intensively studied and reported system despite some limitations. These include the fact that the bystander effect requires cell to cell contact and that deoxythymidine (dT) is a better substrate than GCV for HSV-TK. However, it represents the best example of the evolution of a GDEPT system from studies *in vitro* to the clinic.

This system is also interesting as a method to monitor therapy for the noninvasive imaging, using a gamma camera and single-photon emission tomography (SPET) with ^{131}I-FIAU, of the HSV-TK enzyme (*85*). Furthermore, it may not be necessary to administer GCV systemically. In a mouse transgenic system for HSV-TK, regressions of breast tumours were obtained by direct i.t. administration of GCV (*86*).

The following steps, which are essential for all GDEPT systems, have been demonstrated for the HSV-TK sys-

tems: (1) demonstration *in vitro* of the stability of the transfected/infected tumor cells and their ability to express the encoded enzyme at concentrations high enough to activate the prodrug of choice; (2) demonstration *in vivo* of the viability of the transfected/infected cells, their propensity to generate tumors, and their sensitivity to the selected prodrug; (3) demonstration of a bystander effect; (4) feasibility of the delivery *in vivo* of the suicide gene.

The *in vivo* experimental studies have shown cures for GCV/HSV-TK and have led to at least 15 ongoing clinical trials involving this gene (*87*).

Early studies were undertaken with the GCV/HSV-TK system to substantiate the "tissue mosaicism" strategy, which represents a preventative approach (*72*, *88*). This strategy assumes that genes which modify cellular sensitivity to various anticancer agents could be introduced prophylactically into a tissue so that some cells acquire a given gene while others do not. The result is a mosaic of cells that differ in sensitivity to the anticancer agent under consideration. If a tumor of clonal origin develops later from this tissue, therapy could be conducted at whatever sensitivity the tumor displays, while the host would be protected by those cells in the mosaic normal population that do not share this gene (*88*). This strategy was tested using BALB/c murine cancer cells bearing the HSV-TK gene. Treatment with GCV in clonogenic assays showed that the HSV-TK negative lines were resistant to doses 200- to >1000-fold higher than the HSV-TK positive lines. A bystander effect, called "metabolic cooperation", was shown. Treatment of TK^+-tumor-bearing animals with GCV (150 mg/kg/injection, twice daily, for 5 days), after tumors became visible, induced complete regressions, and long-term survivors were obtained. In contrast, there were no survivors in the control animals with TK^+ tumors that did not receive GCV or in TK^--tumor-bearing animals treated with GCV. Furthermore, animals treated with CP alone, in another chemotherapeutic control, also have no survivors (*88*). These studies were further extended using retrovirus delivery of the HSV-TK gene to murine sarcoma and lymphosarcoma cell lines. Tumors derived from these were sensitive to GCV treatment, and longg-term regressions were obtained in 12 of 19 animals for sarcoma cell lines. Although regressions were seen for sarcoma cells, the tumors regrew and were found to have become TK-negative, a failure attributed to genomic instability (*72*).

More recent studies used a retroviral vector to transduce the HSV-TK gene into preneoplastic murine mammary epithelial cells, to yield a clonal subline sensitized to GCV. GCV treatment of tumors that arose from the transduced cells retarded tumor growth and induced durable regressions in 7 of 20 mice. GCV was ineffective in the controls (*89*). Unfortunately, the mosaicism strategy suffers from the drawback that the gene transfection cannot be confined to a single tissue and is therefore only justified for people with exceptionally high risks of malignancies (e.g., myelodysplastic syndrome) (*72*).

Further attempts focused on generating curative tumor models. Plasmid vectors have been engineered, with the TK gene under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MTV-LTR). These were used to express TK in rat fibroblasts that subsequently became sensitive to ACV (60% cell death at 10 μM and 98% at 100 μM) or to FIAU (>60% cell death at 0.1 μM and 98% at 3.0 μM) (*90*). The cytotoxicity was proportional to the level of HSV-TK expression and dose dependent for ACV and FIAU.

The GCV/HSV-TK system was not as effective in inhibiting human pulmonary adenocarcinoma cells. No bystander effect was observed with TK-positive cells, but confluence was not achieved in these assays (*91*). A number of strategies involve tissue specific expression of HSV-TK, using promoter and enhancer sequences of a variety of genes (see also section 2.3).

The choice of prodrug for a given HSV-TK system is an important one. In murine and rat HCC retrovirally transduced with the HSV-TK gene, GCV treatment killed the cells at concentrations lower than the plasma level attainable in clinical trials. By contrast, ACV was less effective in GDEPT (*92*).

Recently, random sequence mutagenesis was used to create mutant genes of HSV-TK that render cells more sensitive to specific nucleotide analogues than cells expressing wild-type HSV-TK (*77*). Twenty-six HSV-TK mutants were obtained that made cells more sensitive to GCV, and 56 made cells more sensitive to ACV. Only 6 of the mutants gave increased sensitivity to both compounds. Using these genes, stable mammalian cell transfectants were obtained that were 43-fold more sensitive to GCV and 20-fold more sensitive to ACV than cells expressing wild-type HSV-TK (*77*).

The HSV-TK system has been tested in numerous tumor models to date. One of the earliest of these was in brain tumours, which are an interesting model for GDEPT for the following reasons: the brain represents an immunologically privileged site; the tumors are localized to the CNS; retroviral vectors integrate only into dividing cells, therefore the majority of the cells in the brain will not be infected; and GCV is able to cross the blood−brain barrier and tumor destruction is not immune-mediated (*22*).

In these *in vivo* studies, numerous long-term survivors have been described and tumor regressions have also been documented (*93*−*102*) (see Table 3).

HSV-TK has also been used successfully in animal model HCCs, using the AFP promoter to express HSV-TK (*56*−*58*); it has been employed for treatment of metastatic liver disease in conjunction with retroviral delivery, which does not infect normal hepatocytes (*80*), for the treatment of melanoma, where the tyrosinase promoter directed the expression of HSV-TK in the melanocytes (*73*, *74*), and in a wide variety of other models (*103*−*105*).

GCV/HSV-TK is perceived to be safe for patient studies since retroviral producer cells are likely to be destroyed by the GCV treatment, and coupled with the success of the *in vitro* and animal studies, clinical studies are currently proceeding in brain tumors (nine studies), melanoma (one study), head and neck tumors (one study), and ovarian carcinomas (two studies) (*87*).

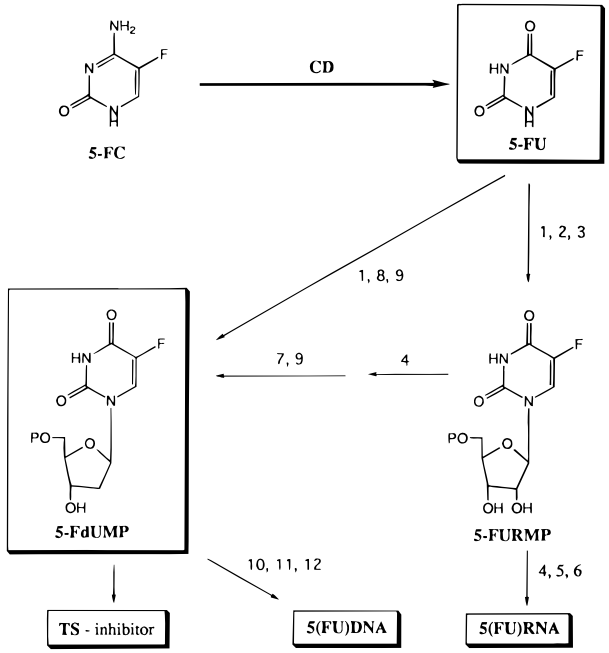
3.3. 5-Fluorocytosine (5-FC)/*E. coli* Cytosine Deaminase (CD). CD is an *E. coli* enzyme, which has homology to human cytidine deaminase but different substrate specificity. CD converts the nontoxic 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to a potent pyrimidine antimetabolite. Its cytotoxicity depends upon cellular anabolism to 5-fluorodeoxyuridine-5′-monophosphate (5-FdUMP) and then to 5-fluorouridine-5′-monophosphate (5-FUMP) by endogenous enzymes (*106*, *107*). 5-FdUMP is a powerful inhibitor of thymidylate synthase (TS) through the formation of the covalently linked ternary enzyme−inhibitor−cofactor complex, resulting in an irreversible inhibition of TS, a key enzyme for DNA biosynthesis and leading to cell death (see Scheme 4). 5-FUMP is phosphorylated to 5-fluorouridine-5′-triphosphate (5-FUTP),

Table 3. Studies on HSV-TK/GCV Gene Therapy in Animal Brain Tumor Models

no.	ref	type of tumor cells	no. of tumor cells	type of viral vector	no. of packaging cells	virus titer (cfu/mL)	% of tumor cells expressing transgene	treatment schedule: GCV (mg/kg/day × days)	50% survival with GCV (days)	50% survival untreated (days)	% long time survivors[a]
1	93	9L	4×10^4	retrovirus	3×10^6			300×5			several animals survive >100 days; *
2	97	9L	4×10^4		1.8×10^6	5×10^5	30−70 (estimatedby LacZ transfer)	30×14			38^+
		9L	4×10^4	retrovirus	3×10^6	5×10^5		30×14			72^+
		9L	4×10^4		5×10^6	5×10^5		30×14			83^+
3	95	9L	2×10^5	retrovirus	2×10^5			50×7	30	22	22^{**} (7/32)
					(2×10^6)			50×14			
4	98	9L	2×10^5	retrovirus	3×10^6	1×10^6	–	90×5			0
5	99	9L	1×10^5	retrovirus	5×10^5	7.5×10^5	45	30×10	35	20	0
6	100	C6	1×10^4	adenovirus		2.5×10^8 (pfu)	–	50×14	101 ± 26	25.4 ± 1.8	
7	101	9L	4×10^4	retrovirus	1.5×10^6		0.1−10.5	30×10	25.9 ± 0.9	24.0 ± 0.4	0
					6×10^6						
		9L	4×10^4	retrovirus			100^+	30×10	60	24.0 ± 0.4	26 (>150 days)
		9L	$4 \times 10^{4(++)}$	retrovirus			100^+	30×10		24.0 ± 0.4	67 (>270 days)
8	102	9L	4×10^4	adenovirus		1×10^8 (pfu)	–	$(2 \times 15) \times 10$		20	10 (at day 80)
		9L	4×10^4	adenovirus		5×10^8 (pfu)	–	$(2 \times 15) \times 10$		20	30 (at day 80)
		9L	4×10^4	retrovirus	5×10^5		–	$(2 \times 15) \times 10$	?	20	20 (at day 80)

[a] *, 11 of 14 animals were free of tumors after treatment; +, % animals with no tumors at the end of the treatment; **, >90 days; +, *ex vivo* transfection; ++, the transfected cells were implanted sc.

Scheme 4. Metabolism of 5-FC[a]



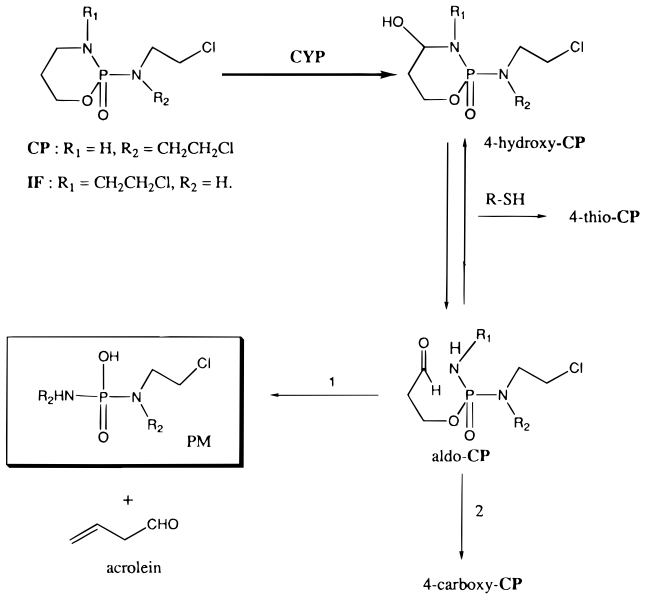
[a] 1, nucleoside phosphorylases; 2, uridine kinase; 3, phosphoribosylpyrophosphate-dependent orotate phosphoribosyltransferase; 4, UMP-kinase; 5, nucleotide diphosphate kinases; 6, DNA polymerase; 7, ribonucleoside reductase; 8, thymidine and/or deoxyuridine phosphorylases; 9, deoxyuridine kinase; 10, dUMP-kinase; 11, (deoxy)nucleoside kinases; 12, DNA polymerase; R-1-P, ribose-1-pyrophosphate; dR-1-P, deoxyribose-1-pyrophosphate.

resulting in the formation of "fraudulent" (5-FU)RNA. This affects RNA maturation and processing and may sometimes be an essential event for cytotoxicity. 5-FUMP is also converted into the corresponding triphosphate (5-FdUTP), by diphosphate kinase, which is incorporated into DNA, interfering with its biosynthesis.

The CD gene has and unusual protein synthesis initiation codon, GTG, and when this is mutated into an ATG, its expression in eukaryotic cells was considerably enhanced (*108*). Murine cells expressing CD with the altered initiation codon showed increased sensitivity to 5-FC, although in this study no bystander effect was seen. In human glioblastoma cells expressing CD, by contrast, a bystander effect was observed with 5-FC, and 5-FU could be detected in tissue culture medium of these cells. Uptake studies revealed a moderate and nonsaturable accumulation of 5-FC in tumor cells supporting its entry by passive diffusion, but a rapid efflux has also been demonstrated, suggesting a possible mechanism for the bystander effect and the reason for the accumulation of 5-FU in tissue culture medium (*109*).

Colorectal carcinoma models have been developed *in vivo* to test 5-FC/CD as a possible novel therapy for metastatic disease in the liver. This is suggested as an innovative alternative therapy since 5-FU is used as chemotherapeutic agent for colorectal carcinoma. Some of these models have recently been reviewed (*9*). In one such system both nonmetastatic colon carcinoma cells and fibrosarcoma cells expressing CD demonstrated dose-dependent sensitivty to 5-FC. In immune competent mice, these cell lines all produced tumors, but treatment with 5-FC was able to give significant tumor regressions. However, different rates of regression were observed (6−47%), depending on the parental tumor line used, indicating that tumor cells differ greatly in their sensitivity to the 5-FC/CD system. Intriguingly, the animals that

Scheme 5. Metabolism of Cyclophosphamide and Ifosfamide[a]



[a] 1, 3′,5′-exonucleases; 2, aldehyde oxidase; R-SH, endogenous SH compounds.

survived the experiment were found to be resistant to subsequent rechallenge with the unmodified parental cell line used, indicating that long-term immune responses had been induced.

Another study with 5-FC/CD has indicated the efficacy of this treatment for colorectal carcinoma. Expression of CD increased the sensitivity of WiDr cells to 5-FC *in vitro*, by ~1000 fold (from IC_{50} = 26 mM to 27 μM), while the expression of CD in these cells did not affect their sensitivity to 5-FU (IC_{50} = 5 μM) (*110*).

In mice bearing xenografts expressing CD, 5-FU concentration in the tumors, of >400 μM, could be generated following 5-FC administration and 5-FU could be detected in the environment surrounding the tumor (*111*). The liberated 5-FU may account for the bystander effect seen in this studies, where it was found that only 2% of tumor cells were needed to express CD to obtain significant regressions following 5-FC administration. These studies have also addressed dose scheduling and found that appropriate dosing could give cure rates of up to 75% in tumor-bearing mice.

Further studies were conducted in attempts to target CD expression to human models, using promoters [c-erb B2 and CEA (*66*, *69*)], adenoviral vectors (*112*), and recently *Clostridium* bacteria, which grow specifically in hypoxic regions of tumors (*18*). Although these studies have met with encouraging success, they have also shown that whereas 5-FC/CD is effective against colorectal cancers (*110*) and lung adenocarcinoma (*91*), disappointing results were obtained with breast and pancreatic cancer models.

3.4. Oxazaphosporines [Cyclophosphamide (CP), Ifosfamide (IF)]/Rat Liver Cytochrome P450 Isoenzyme (CYP2B1) or Human Isoenzyme (CYP2B6). CP and IF are good candidates for GDEPT since they are prodrugs and their biochemical and pharmacological properties are known. CP is activated in rat liver by microsomal CYP to 4-hydroxy-CP, which exists in equilibrium with its ring-opened tautomer aldophosphamide (*113*) as shown in Scheme 5.

This reaction requires NADH and O_2, and its K_M = 0.5−1.5 mM (*113*). There is evidence that the significant circulating and pharmacologically important metabolite is 4-hydroxy-CP/aldo-CP. The cytotoxic metabolite is the corresponding phosphoramide mustard, which is generated together with acrolein from the decomposition of aldo-CP (see Scheme 5). The spontaneous β-elimination of acrolein from aldo-CP is a base-catalyzed process (involving hydroxide ions, carbonate anion, and organic or inorganic phosphate) (*113*). There are, however, data suggesting that 3′,5′-exonucleases (and especially those related to DNA polymerase) catalyze the release of the phosphoramide mustard, and this could be the basis of its relative specificity to the cancer cells (*114*). The activation of IF occurs in a similar way (*115*). These bifunctional alkylating agents kill both cycling and noncycling cells by cross-linking DNA.

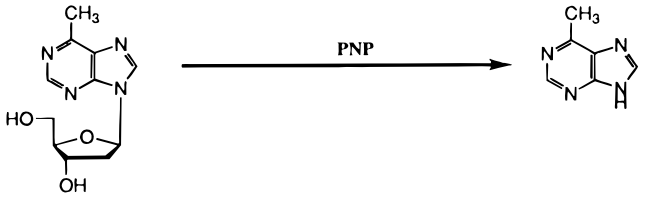
CYP is a complex enzyme of which there are many isoenzymes with different substrate specificities. Not all of the isoenzymes are equally effective in activating CP or IF. Isoenzymes 2B1 (CYP2B1, phenobarbital inducible), 2C6 (CYP2C6), and 2C11 (CYP2C11) (the latter two are constitutively expressed) are the enzymes involved in activating oxazaphosphorines in rats. Isoenzymes 2B6 (CYP2B6) and 3A4 (CYP3A4, expression restricted to liver) are responsible for this process in humans (*116*). CYP2B6 and CYP3A4 are not found in tumor cells. Glioma cell lines expressing CYP2B1 were found to be sensitive to both CP (IC_{50} = 70 μM) and IF (IC_{50} = 145 μM), whereas the parental lines were resistant to millimolar concentrations of these prodrugs. The difference in the IC_{50} figures of CP and IF is in good agreement with the kinetic data, which showed that the CYB2B1 enzyme cleaves IF with an efficiency (V_{max}/K_M) 3−4-fold lower than that of CP. In xenograft models in rats tumors expressing CYP2B1 were found to be sensitive to subsequent CP administration. After 7−8 weeks, none of the rats bearing non-CYP2B1-expressing tumors had survived, whereas 19 of 20 of the animals carrying CYP2B1-expressing tumors were still alive. In these experiments a bystander effect was seen.

The CP/CYP2B1 system has also been shown to be effective in murine glioma models (*117*). In this system, an important observation was that the CP treatment was effective against the tumors, and there was no apparent host toxicity, despite significant levels of isoenzyme capable of activating CP present in normal liver. It should be noted, however, that since the activating enzyme in liver, CYP2C6, is distinct from CYP2B1 used in the therapy, potential problems of activation of CP by liver enzymes could be overcome by the use of CYP-isoform specific inhibitors such as 21,21-dichloroprogesterone or 3,5-dimethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (*118*, *119*).

Retroviral and adenoviral vectors have been used to transduce CYP2B1 into established intracerebral gliomas in rats resulting in significantly prolonged survival of the animals after treatment with prodrugs (*120*). When compared to GCV/TK, the CYP/CP system was found to be less efficient, possibly due to poor penetration of CP across the blood−brain barrier or low viral titers (*120*).

In addition to glioma models, expression of CYP2B1 in other tumor cell types (breast and prostate cancer lines) has been shown to increase the sensitivities of these cells to CP and IF, *in vitro* (*121*). These sensitivities to CP and IF can be reversed by metyrapone, a CYP2B1-selective enzyme inhibitor. Intratumoral CYP2B1 expression conferred a distinct therapeutic advantage in the treatment of MCF-7 (human breast) xenografts in nude mice. CYP2B1-expressing tumors were found to be 15−20-fold more sensitive to appropriate prodrugs than

Scheme 6. Metabolism of 6-MePdR[a]



[a] PNP, purine nucleotidephosphorylase.

the nonexpressing controls (*121*). Encouragingly, the therapeutic benefit was obtained without apparent increases in host toxicity.

3.5. 6-Methylpurine-2′-deoxyribonucleoside (6-MePdR)/*E. coli* Purine Nucleoside Phosphorylase (PNP). PNP (or purine nucleoside orthophosphate transferase) catalyzes the reversible phosphorolysis of purine ribonucleotides (*122*). These enzymes are found in bacteria and in eukaryotes, but there are important differences in substrate specificity between the bacterial and the human enzymes. For instance, adenine is a good substrate for bacterial PNP but not for the human enzyme. Purine arabinosides have at least 10-fold greater activity with *E. coli* PNP compared to the human enzyme. On the other hand, mammalian PNP is more efficient in the synthesis of nucleosides from 2-oxo-6-substituted purines and their analogues than is the bacterial PNP.

In GDEPT, the bacterial PNP was used to activate the relatively nontoxic 6-MePdR to the toxic 6-methylpurine (6-MeP) (see Scheme 6). In human colonic carcinoma cells, 6-MeP was ~100-fold more toxic than 6-MePdR (*123*). When these cells were engineered to express *E. coli* PNP, their sensitivity to 6-MePdR was found to increase 60-fold. Furthermore, a significant bystander effect has been shown, with <1% of cells expressing *E. coli* PNP resulting in the death of virtually all of the cells in the population following prolonged 6-MePdR treatment (*123*).

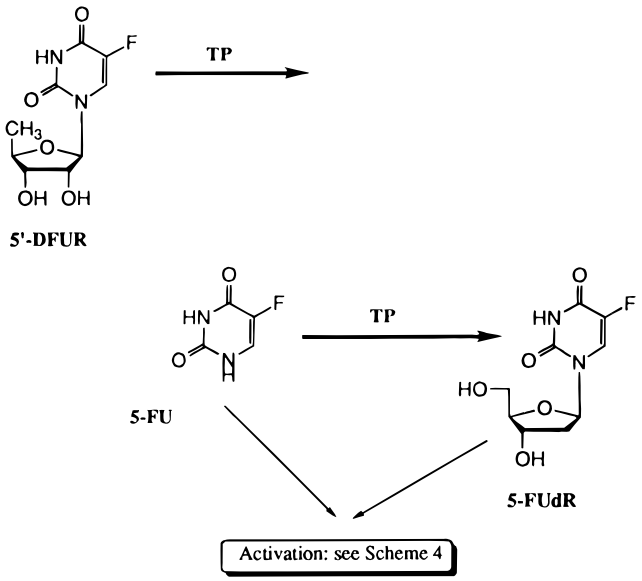
This system has been tested in human melanoma cell lines, using the tyrosinase promoter to direct melanocyte expression (*75*). Human colonic carcinoma cells were used as a control for the promoter. The melanoma cells were sensitized to 6-MePdR, whereas the control cells were not. Furthermore, using murine melanoma cell lines, *in vitro*, complete cell kill could be achieved with only 2% of cells expressing *E. coli* PNP under the control of the SV40 promoter, following long incubation with prodrug.

Other adenine nucleosides such as 2-fluoroadenine, 2-azaadenine, and 4-aminopyrazolo[3,4-*d*]pyrimidine are also potential prodrugs for bacterial PNP and, indeed, tumor cell sensitization to arabinofuranosyl-2-fluoroadenine has been demonstrated using this enzyme (*75*).

3.6. 5′-Deoxy-5-fluorouridine (5′-DFUR)/Thymidine Phosphorylase (TP). Human TP has been proposed for GDEPT in connection with the activation of 5′-DFUR (*124*). TP catalyzes the reversible phosphorolytic cleavage of deoxythymidine, deoxyuridine, and some of their analogues to the corresponding bases and deoxyribose-1-phosphate. (See Scheme 7.) Although TP is implicated in the activation of 5′-DFUR, there has been no clear demonstration that TP of human origin can cleave the glycosidic bond of 5′-DFUR to yield 5-FU.

Nevertheless, it was shown that overexpression of human TP in human breast cancer cells led to a ~90-fold increase in enzyme levels and a concomitant 165-fold increase in sensitivity to 5′-DFUR. The sensitivity of the cells to 5-FU and 5-FUdR remained unchanged (*124*).

Scheme 7. Metabolism of 5′-DFUR[a]



[a] TP, thymidine phosphorylase.

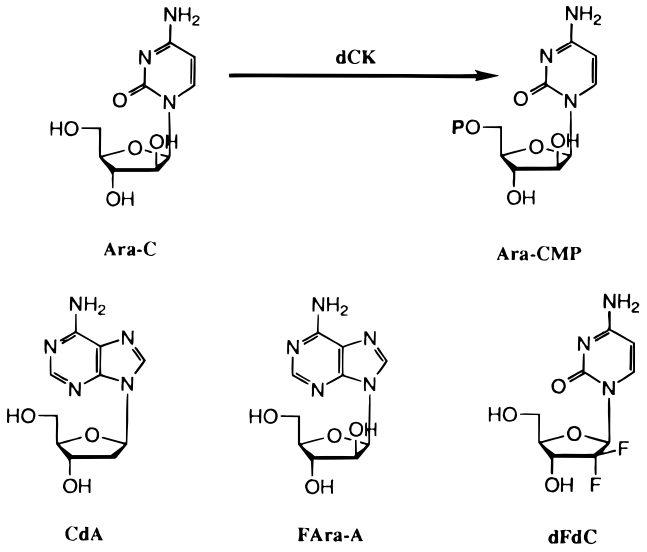
3.7. Arabinosylcytosine [1-(β-D-Arabinofuranosyl)cytosine (Ara-C), 2-Chloro-2′-deoxyadenosine (CdA), 2-Fluoro-9-(β-D-arabinofuranosyl)cytosine (Fara-C), 2-Fluoro-9-(β-D-arabinofuranosyl)adenine (Fara-A), and 2′,2′-Difluorodeoxycytidine (dFdC)/Deoxycytidine Kinase (dCK)]. Human dCK is an enzyme involved in the salvage pathway of deoxyribonucleotides biosynthesis. The phosphorylation reaction is the limiting step in this pathway (*125*). The prodrugs ara-C, CdA, Fara-C, Fara-A, and dFdC can all be activated by a phosphorylation reaction to the corresponding deoxynucleotide monophosphates which, after further phosphorylation to the corresponding triphosphates, are biosynthetically incorporated into RNA and DNA, leading to DNA fragmentation and consequent cell death. (See Scheme 8.)

There have been suggestions that tumor sensitivity to these drugs correlates with dCK activity, but since tumors have relatively low levels of dCK, it has been proposed that dCK-gene transfer will be useful for chemotherapy.

To investigate this proposition, human colon carcinoma and small-cell lung adenocarcinoma cell lines have been engineered to express the human dCK-protein. MCF-7 cells expressing dCK showed a 2.5-fold increased sensitivity to ara-C, whereas H1437 cells expressing dCK displayed 106-fold increase to ara-C. In these studies a linear correlation between IC_{50} and dCK activity was suggested (*125*).

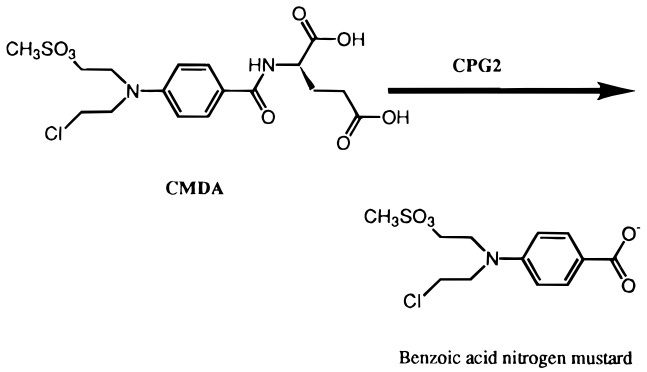
The ara-C/dCK system represents a good model for brain tumors, since ara-C can cross the blood−brain barrier and the activated drug is not toxic to postmitotic cells. Glioma cells expressing dCK were ~10-fold more sensitive to ara-C than parental cells, although this was observed following 4 days of continuous exposure (*126*). The bystander effect, which requires cell to cell contact, was disappointing, with only 50% cell kill occurring when 10% of the cells expressed dCK. In spite of these results, six of seven rats with intracerebral gliomas that expressed dCK were cured (survival >120 days, in comparison with control animals, which were dead after 19−

Scheme 8. Activation of Ara-C by dCK and Other Nucleosides That Could Be Activated by the Same Enzyme[a]



[a] dCK, deoxycytidine kinase.

Scheme 9. Activation of CMDA by the CPG2 Enzyme[a]



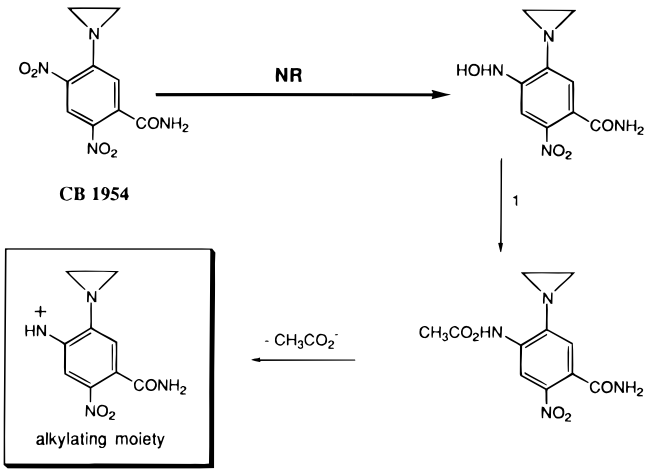
[a] CPG2, carboxypeptidase G2.

29 days). Furthermore, the dCK gene was transduced into established intracerebral glioma tumors using adenoviral vectors which, following ara-C treatment, resulted in significantly longer survival than found for animals treated with the control adenoviral vector plus ara-C.

3.8. (2-Chloroethyl)(2-mesyloxyethyl)aminobenzoyl-L-glutamic Acid (CMDA)/*Pseudomonas* Sp. Carboxypeptidase G2 (CPG2). CPG2 is a bacterial enzyme from a *Pseudomonas* species (*127*) for which there are no known human homologues, and it is therefore a good candidate for GDEPT. CPG2 catalyzes the scission of amidic, urethanic, and ureidic bonds between an aromatic nucleus and L-glutamic acid (see Scheme 9) (*128*−*130*). It can therefore be used to activate prodrugs such as CMDA to aromatic bifunctional alkylating agents.

CMDA, a benzoylglutamate mustard, is cleaved by CPG2 to a benzoyl mustard which can cross-link DNA. Since no endogenous enzymes are required for this activation, the chance of developing resistance is greatly reduced. The released drug is also active against cycling and nonncycling cells and therefore has an advantage over antimetabolite toxins (e.g., GCV or 5-FC). CPG2 has been expressed stably in a variety of human tumor cell lines (breast, ovarian, and colon carcinomas) which rendered the cells sensitive to the prodrug CMDA. The cell lines expressing CPG2 were found to be 10−100

Scheme 10. Metabolism of CB 1954[a]



[a] NR, nitroreductase; 1, acetyl-coenzyme A.

times more sensitive to CMDA than appropriate controls *in vitro*. Total cell kill could be achieved when 4−12% of the cells expressed CPG2, depending on the cell line tested (*8*).

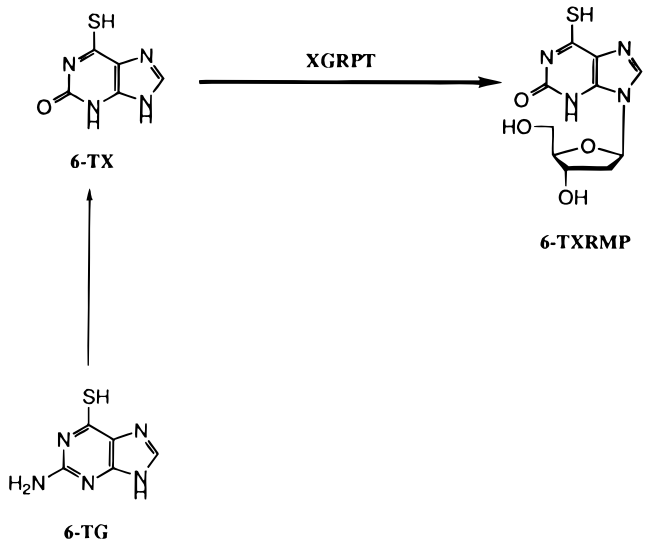
3.9. 5-Aziridinyl-2,4-dinitrobenzamide (CB 1954)/*E. coli* Nitroreductase (NR). The prodrug CB 1954, a monofunctional alkylating agent, was found to be extremely potent against the Walker carcinosarcoma in rats but inactive against a panel of tumors used in screening anticancer agents (*131*). It has subsequently been shown that the sensitivity of the Walker cell line was due to DT-diaphorase present in these cells, which activates CB1954 into the bifunctional alkylator 5-aziridinyl-4-(hydroxylamino)-2-nitrobenzamide (*132*, *133*). This is probably converted by acetyl-CoA or other endogenous enzymes to a more potent electrophile, for example, the corresponding acetate, which then reacts with DNA. The metabolism of CB 1954 is shown in Scheme 10. Since the active drug is an alkylating agent, it is able to kill both cycling and noncycling cells, an advantage compared to antimetabolite toxins. Human DT-diaphorase is much less able to activate CB 1954 than is the rat enzyme and therefore should not pose a problem for the use of CB1954 in patients.

Bacterial NR is also able to activate CB 1954, and NR is much more effective than human DT-diaphorase in activating the prodrug (*134*). A recent study found that expression of *E. coli* NR in NIH3T3 cells increased their sensitivity to NR. Some co-operative killing was observed when cells expressing both NR and TK were treated with a combination of CB 1954 and GCV, supporting their different mechanisms of action (*7*).

3.10. 6-Thioguanine (6-TG) and 6-Thioxanthine (6-TX)/*E. coli* Xanthine−Guanine Phosphoribosyltransferase (XGPRT). Bacterial XGPRT and its human counterpart (HPRT) catalyze the conversion of hypoxanthine and guanine to inosine monophosphate (IMP) and guanosine monophosphate (GMP), respectively. XGPRT also efficiently converts xanthine to XMP, a reaction that is poorly catalyzed by the human enzyme (*135*). These nucleoside monophosphates serve as substrates for pathways involved in the biosynthesis of DNA and RNA. The bacterial enzyme is also able to transfer the ribose phosphate moiety to 6-TX and 6-TG. (See Scheme 11.)

XGPRT is encoded by the *E. coli* gpt gene (*135*, *136*). XGPTR expression in mammalian cells can be used either as a positive or negative selection depending on the

Scheme 11. Activation of 6-TX and 6-TG by XGRPT[a]



[a] XGRTP, xanthine−guanine phosphoribosyltransferase.

prodrug used (e.g., hypoxanthine and xanthine positively select cells that express XGPRT, whereas 6-TX kills these cells) (*136*). 6-TG can also be used, despite the fact that it is not a XGPRT substrate, since 6-TG is metabolized to 6-TX by endogenous enzymes, which are subsequently activated by XGPRT.

Clonal glioma lines expressing XGPRT showed significantly increased sensitivity to 6-TX (IC_{50} = 2.5 μM/L; parental line IC_{50} > 50 μM/L). The XGPRT expressing cell line was also sensitive to 6-TG (IC_{50} = 0.05 μM/L, parental line IC_{50} = 0.5 μM/L). Cell to cell contact was shown to be required for bystander effects, and under these conditions, significant bystander effects were seen when ~11% of cells expressed XGPRT. Both 6-TG and 6-TX significantly inhibited the growth of tumors in nude mice formed from the sc or intracerebral (ic) transplantation of XGPRT expressing cells.

3.11. Design of Prodrugs for GDEPT. The design of prodrugs for GDEPT should address several issues related to clinical situations. These include the following: penetration of prodrugs into cancer cells for intracellular activation; low toxicity of prodrugs; effective activation of prodrug by the expressed enzyme with favorable activation kinetics; high cytotoxicity of the active drug; ideally, the released drugs should be effective in both cycling and noncycling cells; and the released drugs should induce a bystander effect.

Lipophilic prodrugs are required to penetrate across cell membranes, or prodrugs could be synthesized that are taken up by active transport. It appears that the prodrugs so far in use (nucleoside analogues, 5-FC, CP, CMDA, and CB 1954) penetrate cells by passive diffusion as opposed to active uptake. Low cytotoxicity for the prodrugs is essential since they will also be taken up by normal cells. Activation of the prodrugs is one of the critical steps in GDEPT. It is an advantage if prodrug activation proceeds directly by the expressed enzyme, without the requirement for further catalysis by endogenous enzymes. The involvement of host enzymes in prodrug activation may lead to resistance of tumor cells if the endogenous enzymes are deficient or defective. Furthermore, cells may differ in the levels of the endogenous enzymes they express and thus individual tumors may differ in their sensitivity to prodrugs, even if they express equivalent amounts of exogenous protein. With ara-M, GCV, and ACV the toxic metabolites are generated by mammalian adenosine monophosphate deaminase, AMP- and/or GMP-kinases following initial activation by TK (see Schemes 2 and 3). With 5-FC, which is converted to 5-FU by CD, the active metabolite is 5-FdUMP or 5-FUTP, which results from 5-FU conversion by a number of mammalian enzymes involving a complicated activation pattern (see Scheme 4). The complex activation pathway of these antimetabolites is partially responsible for their propensity to induce resistance (*137*).

The prodrugs CP, IF, and CB 1954 are dependent on fewer endogenous enzymes for activation than those cited above. CP, after the primary activation by CYP, requires a second step by 3′,5′-exonuclease to generate the active metabolite. For CB 1954, the 5-aziridinyl-4-hydroxylamino-2-nitrobenzamide intermediate resulting from the prodrug reduction by NR needs a further enzymatic step to be converted to the electrophile which alkylates DNA. With the alkylating agent prodrug CMDA, catalysis by CPG2 leads directly from the prodrug to the activated drug (see Scheme 9). These prodrugs, theoretically, have less opportunity to generate resistance.

It is an advantage if the prodrugs are activated to drugs with high cytotoxicity and optimum half-lives to maximize the benefits for GDEPT. The half-lives should be short enough not to allow the drug to leak out from the tumor but long enough to induce a bystander effect. Good kinetic parameters (higher k_{cat}) seem favorable. This effect was noticed when the activity of CP (higher k_{cat}, lower IC_{50}) was compared with that of IF (lower k_{cat}, higher IC_{50}) (*138*). Also, as described above, improving the kinetic parameters of TK by mutagenesis has resulted in enzymes that make cells more sensitive to prodrugs than wild-type enzymes (*77*).

Ideally the released drug should be effective against both cycling and noncycling cells. Unfortunately, the majority of prodrugs used in GDEPT release active drugs that are antimetabolites, which require cycling cells (S phase) for cytotoxicity. It has been speculated that the resistance to GCV in GDEPT is not an acquired one but resulted from tumor cells being in G_0 during the time of GCV administration (*139*). This argument is supported by the fact that the tumors which grew out remained sensitive to GCV. The use of prodrugs that are activated to alkylating agents should have an advantage over purine nucleosides or 5-FC, since they are cytotoxic to both cycling and noncycling cells and this has been shown to be true for the alkylating agent CB 1954 (*7*). Alkylating agents also have the advantage that they are less prone than other anticancer compounds to induce resistance (*140*, *141*).

4. BYSTANDER EFFECT AND ACQUIRED IMMUNITY

Since expression of the foreign enzymes will not occur in all cells of a targeted tumor *in vivo*, a bystander cytotoxic effect is required whereby the prodrug is cleaved to an active drug that kills not only tumor cells but also neighboring nonexpressing tumor cells (*111*). This means that expression in <100% of tumor cells can still give total tumor cell kill. It was demonstrated, *in vivo*, that when as few as 2% of the tumor cells expressed the foreign enzyme, subsequent treatment with suitable prodrug gave long-term animal survivors (*111*). Therefore, an expression efficiency of 10−20% should be enough to achieve 100% cell kill in tumors, and efficiencies of 1−5% are considered sufficient for a therapeutic benefit (*111*). Even with lower transfer efficiencies,

beneficial results were obtained if the transduced cell population was allowed to expand before prodrug administration.

The systems reported have exhibited a significant bystander effect *in vivo*. However, the mechanisms may be different for individual drugs. For instance, for the toxic nucleoside phosphates resulting from the activation of purine (GCV, ACV, etc.) or pyrimidine (ara-C, 5-FUDR, etc.) nucleosides, which do not diffuse across cell membranes, cell to cell contact is required to obtain the bystander effect (*142*). By contrast, for diffusible drugs such as 5-FU or aldophosphamide (or phosphoramide mustard) no such requirement is necessary (see below) (*111, 138*).

The responses described in GDEPT would surely not be possible without the existence of the bystander effect. It is very difficult to compare bystander effects obtained in different experimental models due to the different conditions employed by the various investigators. However, we have summarized the data on the bystander effects in different systems in Table 4.

The bystander effects vary depending not only on which prodrug is combined with which enzyme but also on whether the effects are monitored with *in vitro* or *in vivo* systems. It is also clear that different tumor cell types give different results even within the same prodrug/enzyme system. For example, using CMDA with CPG2 *in vitro* in the colorectal carcinoma cell line WiDr, 12% transfected cells were required for total cell kill, whereas with the colorectal carcinoma cell line LS174T only 4% were needed to give 100% cytotoxicity (*8*). With the GCV-TK combination, 25% of Ov-Ca-1225 TK expressing cells were required for total cell kill *in vitro*. In a liver carcinoma cell line, 10−20% of cells expressing TK were needed for total regression of tumors (*80*).

The mechanisms responsible for the bystander effects are not completely understood. Several explanations are plausible, and there is the possibility that more than one mechanism is involved. It is also plausible that the mechanisms are different when different types of prodrugs are used. In addition, mechanisms *in vivo* are different from those *in vitro*.

Toxic metabolites are formed during prodrug activation. These are released by efflux from dead and dying genetically modified cells. This is postulated for 5-FU formed from 5-FC, for the metabolites of CP or IF, aldophosphamide, phosphoramidic mustards, or acrolein (*7, 111, 138*), and for 6-MeP, formed from the corresponding deoxynucleoside (*123*). This assumption is supported by the fact that no cell to cell contact is required to obtain a bystander effect in these systems. In WiDr cells, this effect was observed both *in vitro* and *in vivo*. *In vitro*, 30% of cells expressing CD were required to eradicate the whole cell population (*111*). However, *in vivo* the bystander effect was more dramatic and 2% WiDr-CD cells were enough to obtain 100% tumor regression in nude mice, while 4% of cells expressing CD resulted in cures in 66% of the animals (*91, 111, 143*). When similar experiments were performed in immunocompetent animals, the results were better, indicating that there is a significant immune component to GDEPT.

For purine or pyrimidine nucleosides, where the toxic metabolites, being phosphorylated, are not diffusible across cell membranes, direct cell to cell contact is required to achieve a bystander effect (*142*). When TK^+ cells were separated by a 0.4 μm filter membrane from TK^- cells, the bystander effect was abrogated. A similar mechanism could also be postulated for other systems involving different purine or pyrimidine nucleotides as active drugs, for instance ara-C/dCK (*126*), 6-TA, 6-TX/XGPRT (*136*), or 5′-DFUR/TPP (*124*). Since purine and pyrimidine nucleotides formed during activation cannot cross cell membranes, it was assumed that gap junctions are involved in the transfer of toxic metabolites from cell to cell (*142, 144*). Another suggestion is that the TK-enzyme itself is transported by apoptotic vesicles or through gap junctionss (*142, 145, 146*). Using a flow cytometry assay, it has been shown (in a coculture of HSV-TK expressing murine fibroblasts with rat glioma cells or human hepatoma cells) that bystander tumor cytotoxicity during GCV treatment was highly correlated ($p < 0.001$) with the extent of gap junction mediated coupling, supporting their role in mediating the bystander effect (*147*).

Phagocytosis by the nonenzyme-expressing cells of materials other than the active drug or the activating enzyme from the dying cells (e.g., hydrolases or other lytic enzymes) has been suggested as a mediator of the bystander effect (*142, 148*). However, in contrast with this theory, during the period of apoptosis, in bystander cells cocultured with HSV-TK-expressing cells, no phagocytosis could be observed (*149*). Apoptosis has been shown in bystander cells and was found to be inhibited by bcl2 expression supporting a role for apoptosis in bystander events (*149*). Furthermore, it has also been suggested that killing of tumor cells by apoptosis could heighten the immune response to wild-type tumor cells (*142*).

There have been suggestions that the immune response is a favorable event in GDEPT. Although data are available which show that the bystander effect also occurs in immunoincompetent animals, more recent findings suggest that the bystander effect *in vivo* can be mediated through the release of cytokines (*148, 150*). Coinjection of mouse colon carcinoma cells with HSV-TK-expressing retroviral packaging cells followed by GCV treatment resulted in almost total tumor ablation in immunocompetent BALB/c mice but not in immunocompromised athymic BALB/c animals (*79*). In another study, HSV-TK-expressing 3T3, which were transformed by Ki-Ras V12G, were shown to be sensitive to GCV. Tumor growth with these cells was inhibited for up 50 days following GCV treatment in immunocompromised nude mice, but this was not sufficient to eliminate all of the tumor cells in these animals (tumors regrew 40−50 days after cell implantation). By contrast, in immunocompetent BALB/c mice, this treatment led to the development of a long-lasting immunity (*151*). Similarly, in experimental metastasis of murine melanoma models, a greater reduction in the number of metastatic sites was seen in immunocompetent mice than in nude mice, indicating an immune component to the bystander effect (*152*).

The role of IL-2 in these systems has also been investigated. Syngeneic colon carcinoma cells grown in BALB/c mice were injected with an adenoviral vector containing the HSV-TK gene or the IL-2 gene, followed by treatment with GCV. While the tumors continued to grow in the animals injected with a control vector or the vector carrying the IL-2 gene, those treated with HSV-TK, with or without coadministration of IL-2, exhibited tumor necrosis and regressions. However, only animals treated with HSV-TK together with the IL-2 genes developed effective systemic antitumoral immunity against tumorigenic rechallenges. The antitumoral immunity was associated with the presence of tumor cell specific cytolytic $CD8^+$ T-lymphocytes (*153*). To enhance and prolong the antitumoral immunity, a third vector con-

Table 4. Bystander Effect

no.	system	bystander effect (transgenes needed to obtain a certain response)		comments	refs
		in vitro	*in vivo*		
1	herpes simplex virus thymidine kinase/ganciclovir	1% 9L/TK1.3 cells required for 60% cell kill; 25% Ov-Ca-1225 TK^+ cells required for 100% cell kill	50% WiDrTK cells are required for 60% tumor-free animals, day 70; 10−20% cells transfection efficiency is needed in liver tumors for regressions	cell to cell contact required	80, 96, 143
2	cytosine deaminase/5-fluorocytosine	significant bystander effect in H1347 and H2122 cells	4% WiDrCD cells are needed for xenografts cures; 2% WiDrCD cells are needed for tumor regressions and 4% for 66% cures	diffusible active drug	111, 143
3	cytochrome P450/cyclophosphamide, ifosfamide	25% transgenes required for 75% cell kill	significant (no quantitative data)	diffusible active drug	117, 121
4	purine nucleoside phosphorylase/9-(β-D-2′-deoxy-*erythro*-pentafuranosyl)-6-methylpurine	>1% T84-PNP cells (colon cancer) required for 100% cell kill; 1−2% MeI-PNP (human melanoma) required for 100% cell kill		diffusible active drug; prolonged prodrug exposure	75, 123
5	thymidine phosphorylase/5′-deoxy-5-fluorouridine	MCF-7 cell population reduced 10-fold in a mixture containing 20% MCF7-TP cells (estimated on IC_{50} basis)		diffusible metabolite	124
6	deoxycytidine kinase/arabinosylcytosine	10% 9LdCK cells required for 50% cell kill		cell to cell contact required	126
7	carboxypeptidase G2/(2-chloroethyl)-(2-mesyloxyethyl)aminobenzoyl-L-glutamic acid	∼12% WiDr-CPG2 or SK-Ov3-CPG2 cells required for 100% cell kill; 4−5% LS174T-CPG2 or A2780-CPG2 cells required for 100% cell kill		diffusible metabolite	8
	nitroreductase/5-aziridinyl-2,4-dinitrobenzamide	30−50% transgenes needed for 90% cell kill		diffusible metabolite	7
8	xanthine−guanine phosphoribosyl-transferase/6-thioxanthine, 6-thioguanine	complete inhibition of growth at C6GPT:C6 glioma cells ratio of 1:1		cell to cell contact required	136

taining the mouse granulocyte macrophage colony stimulating factor (mGM-CSF) gene was also employed. The animals treated simultaneously with HSV-TK + IL-2 + mGM-CSF vectors, followed by administration of GCV, developed long-term antitumor immunity and survived for >4 months without recurrence (*154*).

The synergism between "suicide gene" and "cytokine gene" therapies has also been tested in head and neck squamous carcinoma in mice. Animals receiving both HSV-TK and IL-2 genes, followed by GCV treatment demonstrated a significantly greater regression in tumours compared to those treated with the components without the cytokine gene (*155*). Taken together, these studies suggest strongly that an intact immune system is important for long term tumour suppression *in vivo*.

5. FUTURE PERSPECTIVES

As described above there are a large number of prodrug/enzyme combinations that have shown efficiency *in vitro* and *in vivo*. There remain some hurdles to overcome before GDEPT will become a clinically efficient treatment of solid tumors. These include the development of better and more efficient delivery systems for the exogenous genes together with restricted expression in the tumour alone; the synthesis of prodrugs that are better substrates for the transfected genes, which are not substrates for endogenous enzymes and that exhibit good bystander effects; and the use of mutated genes that express enzymes with higher specificity for their substrates.

The following steps have already been achieved in GDEPT: transfection of tumor cells with the gene for an enzyme *in vitro* have been able to activate specific prodrugs, converting them to cytotoxic drugs; transfected tumor cells, when injected into animals, are able to grow and to generate tumors, which have subsequently undergone regressions or been cured by administration of appropriate prodrugs; the "bystander effect" has been measured *in vitro* and *in vivo* (in the presence of an immune system, this bystander effect may become long-term); gene transfection has been achieved *in vivo* using direct DNA injection (naked DNA) liposomes, cationic lipids, or gene transduction with adenoviral or retroviral vectors; and gene infection has been achieved *in vivo* using packaging cells that continuously produce the desired retroviral vector.

The demonstration of these steps in combination with the prodrugs described herein illustrates not only the feasibility of the currents systems but also the future promise of GDEPT.

ACKNOWLEDGMENT

Work in this area is funded by the Cancer Research Campaign. We thank Professors Ken Harrap and Chris Marshall for their support.

LITERATURE CITED

(1) W. W. Zhang, T. Fujiwara, E. A. Grimm, and J. A. Roth (1995) Advances in cancer gene therapy. *Adv. Pharmacol. 12*, 289−341.

(2) R. E. Christoffersen and J. J. Marr (1995) Ribozymes as human therapeutic agents. *J. Med. Chem. 38*, 2023−2037.

(3) B. P. Sorrentino, C. J. Brandt, D. Bodine, M. Gottesman, I. Pastan, A. Cline, and A. W. Nienhuis. (1992) Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. *Science 257*, 99−103.

(4) G. Wang, C. Weiss, P. Sheng, and E. Bresnick. (1996) Retrovirus mediated transfer of the human O6-methylguanine-DNA methyl transferase gene into a murine hematopoietic stem cell line and resistance to the toxic effects of certain alkylating agents. *Biochem. Pharmacol. 51*, 1221−1228.

(5) J. E. Reese, O. N. Koc, K. M. Lee, L. Liu, J. A. Allay, W. P. Phillips, and S. L. Gerson (1996) Retroviral transduction of a mutant methylguanine DNA methyltransferase gene into human CD34 cells confers resistance to O6-benzylguanine plus 1,3-bis(2-chloroethyl)-1-nitrosourea. *Proc. Natl. Acad. Sci. U.S.A. 93*, 14088−14093.

(6) S. Letourneau, M. Greenbaum, and D. Cournoyer. (1996) Retrovirus-mediated gene transfer of rat glutathione-S-transferase Yc confers *in vitro* resistance to alkylating agents in human leukemia cells and in clonogenic mouse hematopoietic progenitor cells. *Hum. Gene Ther. 7*, 831−840.

(7) G. Bridgewater, C. J. Springer, R. Knox, N. Minton, P. Michael, and M. Collins. (1995) Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur. J. Cancer 31A*, 2362−2370.

(8) R. Marais, R. A. Spooner, Y. Light, J. Martin, and C. J. Springer (1996) Gene-directed enzyme prodrug therapy with a mustard prodrug/carboxypeptidase G2 combination. *Cancer Res. 56*, 4735−4742.

(9) B. E. Huber, C. A. Richards, and E. A. Austin (1995) VDEPT; An enzyme/prodrug gene therapy approach for the treatment of metastatic colorectal cancer. *Adv. Drug Delivery Rev. 17*, 279−292.

(10) R. G. Vile, R. M. Diaz, N. Miller, A. Tuszyanski, and S. G. Russell (1995) Tissue-specific gene expression from Mo-MLV retroviral vectors with hybrid LTRs containing the murine tyrosinase enhancer/promoter. *Virology 214*, 307−313.

(11) E. J. Kremer and M. Perricaudet (1995) Adenovirus and AAV mediated gene transfer. *Br. Med. Bull. 51*, 31−44.

(12) J. C. Glorioso, N. A. DeLuca, and D. J. Fink (1995) Development and application of herpes simplex virus vectors for human gene therapy. *Annu. Rev. Microbiol. 49*, 675−710.

(13) F. Ledley (1995) Nonviral gene therapy: the promise of genes as pharmaceutical products. *Hum. Gene Ther. 6*, 1129−1144.

(14) G. P. Behr (1994) Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. *Bioconjugate Chem. 5*, 382−389.

(15) M. S. Wadha, W. T. Collard, R. C. Adami, D. L. McKenzie, and K. G. Rice (1997) Peptide-mediated gene delivery: influence of peptide structure on gene expression. *Bioconjugate Chem. 8*, 81−88.

(16) R. A. Spooner, M. P. Deonarain, and A. A. Epenetos (1995) DNA vaccination for cancer treatment. *Gene Ther. 2*, 173−180.

(17) S. W. Ebbinghaus, N. Vigneswaran, C. R. Miller, R. A. Chee-Awai, C. A. Mayfield, D. T. Curiel, and D. M. Miller (1996) Efficient delivery of triplex forming oligonucleotides to tumour cells by adenovirus-polylysine complexes. *Gene Ther. 3*, 287−297.

(18) M. E. Fox, M. J. Lemmon, M. L. Mauchline, T. O. Davis, A. J. Giaccia, N. P. Minton, and J. M. Brown (1996) Anaerobic bacteria as a delivery system for cancer gene therapy: *in vitro* activation of 5-fluorocytosine by genetically engineered clostridia. *Gene Ther. 3*, 173−178.

(19) S. J. Russell (1994) Replicating vectors for gene therapy of cancers: risks, limitations and prospects. *Eur. J. Cancer 30A*, 1165−1171.

(20) S. J. Russell, A. Brandenburger, C. L. Flemming, M. K. Collins, and J. Rommelaere (1992) Transformation-dependent expression of interleukin genes delivered by a recombinant parvovirus. *J. Virol. 66*, 2821−2828.

(21) X. S. Wang, M. C. Yoder, S. Z. Zhou, and A. Srivastava (1995) Parvovirus B19 promoter at map unit 6 confers autonomous replication competence and erythroid specificity to adeno-associated virus 2 in primary human hematopoietic progenitor cells. *Proc. Natl. Acad. Sci. U.S.A. 92*, 12416−12420.

(22) K. W. Culver (1996) The role of herpes simplex thymidine kinase gene transfer in the drug treatment of brain tumors. *CNS Drugs 6*, 1−11.

(23) E. Benaim and B. P. Sorrentino (1996) Gene therapy in pediatric oncology. *Invest. New Drugs 14*, 87−99.

(24) F. Kanai, Y. Shiratori, H. Wakimoto, Y. Kanegae, I. Saito, H. Nakabayashi, T. Tamaoki, T. Tanka, K. H. Lan, N. Kato, S. Shiina, and M. Omata (1996) Gene therapy for α-fetoprotein producing human hepatoma cells by adenovirus mediated transfer of the herpes simplex virus thymidine kinase gene. *Hepatology 23*, 1359−1368.

(25) Y. Takeuchi, F. L. Cosset, P. J. Lachmann, H. Okada, R. A. Weiss, and M. K. Collins (1994) Type-C retrovirus inctivation by human complement is determinated by both the viral genome and the producer cell. *J. Virol. 68*, 8001−8007.

(26) R. P. Rother, W. L. Fodor, J. P. Springhorn, C. W. Birks, E. Setter, M. S. Sandrin, S. P. Squinto, and S. A. Rollins (1995) A novel mechanism of retrovirus inactivation in human serum mediated by anti-α-galactosyl natural antibody. *J. Exp. Med. 182*, 1345−1355.

(27) Y. Takeuchi, C. D. Porter, K. M. Strahan, A. F. Preece, K. Gustafsson, F. L. Cosset, R. A. Weiss, and M. K. Collins (1996) Sensitization of cells and retroviruses to human serum by (α1−3) galactosyl transferase. *Nature 379*, 85−88.

(28) F.-L. Cosset, Y. Takeuchi, J. L. Battini, R. A. Weiss, M. K. Collins, and S. J. Russell (1995a) High-titer packaging cells producing recombinant retroviruses. *J. Virol. 69*, 7430−7436.

(29) R. J. Rigg, J. Chen, J. S. Dando, S. P. Forestell, I. Plavec, and E. Bohnlein (1996) A novel human amphotropic packaging cell line: high titer, complement resistance, and improved safety. *Virology 218*, 290−295.

(30) D. W. Russell, S. M. Berger, and A. D. Miller (1995) The effects of human serum and cerebrospinal fluid on retroviral vectors and packaging cell lines. *Hum. Gene Ther. 5*, 635−641.

(31) K. Shimizu, Y. Miyao, M. Tamura, H. Kishima, M. Okhawa, E. Mabuchi, M. Yamada, T. Hayakawa, and K. Ikenaka (1995) Infectious retrovirus is inactivated by serum but not by cerebrospinal fluid or fluid from the tumor bed in patients with malignant glioma. *Jpn. J. Cancer Res. 86*, 1010−1013.

(32) R. Weiss (1993) In *The Retroviridae* (J. Levy, Ed.) Vol. 2, pp 1−108, Plenum Press, New York.

(33) P. Roux, P. Jeanteur, and M. Piechaczyk (1989) A versatile and potentially general approach to the targeting of specific cell types by recombinant retroviruses. *Proc. Natl. Acad. Sci. U.S.A. 86*, 9079−9083.

(34) M. Etienne-Julan, P. Roux, S. Carillo, P. Jeanteur, and M. Piechaczyk (1992) The efficiency of cell targeting by recombinant retroviruses depends on the nature of the receptor and the composition of the artificial cell-virus linker. *J. Gen. Virol. 73*, 3251−3255.

(35) H. Neda, C. Wu, and G. Y. Wu (1991) Chemical modification on a ecotropic murine leukemia virus results in redirection of its target specificity. *J. Biol. Chem. 266*, 14143−14146.

(36) J. Calafat, H. Janssen, P. Demant, J. Hilgers, and J. Zavada (1983) Specific selection of host cell glycoproteins during assembly of murine leukemia virus and vesicular stomatitis virus: presence of H-2, Pgp-1 and t-200 glycoproteins on the envelopes of these virus particles. *J. Gen. Virol. 64*, 1241−1253.

(37) J. A. T. Young, P. Bates, K. Willert, and H. E. Varmus (1990) Efficient incorporation of human CD4 protein into avian leukosis virus particle. *Science 250*, 1421−1423.

(38) L. O. Arthur, J. V. J. Bess, R. R. Sowder II, R. E. Benveniste, D. L. Mann, J. C. Chermann, and L. E. Henderson (1992) Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science 258*, 1935−1938.

(39) D. W. Russell, R. E. Hawkins, and G. Winter (1993) Retroviral vectors displaying functional antibody fragments. *Nucleic Acids Res. 21*, 1081−1085.

(40) T.-H. T. Chu, I. Martinez, W. Sheay, and R. Dornburg (1994) Cell targeting with retroviral particles containing antibody-envelope fusion proteins. *Gene Ther. 1*, 292−299.

(41) N. V. Somia, M. Zoppe, and I. M. Verma (1995) Generation of targeted retroviral vectors by using single-chain variable fragment: an approach to in vivo gene therapy. *Proc. Natl. Acad. Sci. U.S.A. 92*, 7570−7574.

(42) T.-H. T. Chu and R. Dornburg (1995) Retroviral vector particles displaying the antigen-binding site of an antibody enable cell-type-specific gene transfer. *J. Virol. 69*, 2659−2663.

(43) S. Valsesia-Wittman, A. Drynda, G. Deleage, M. Aumailley, J.-M. Heard, O. Danos, G. Verdier, and F. L. Cosset (1994) Modifications in the binding domain of avian retrovirus envelope protein to redirect the host range of retroviral vectors. *J. Virol. 68*, 4609−4619.

(44) N. Kasahara, A. M. Dozy, and Y. W. Kan (1994) Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. *Science 266*, 1373−1376.

(45) K. Han, N. Kasahara, and Y. W. Kan (1995) Ligand-directed retroviral targeting of human breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A. 92*, 9747−9751.

(46) T. Matano, T. Odawara, A. Iwamoto, and H. Yoshikura (1995) Targeted infection of a retrovirus bearing a CD4-Env chimera into human cells expressing human immunodeficiency virus type 1. *J. Gen.Virol. 76*, 3165−3169.

(47) F.-L. Cosset, F. J. Morling, Y. Takeuchi, J. L. Battini, R. A. Weiss, M. K. Collins, and S. J. Russell (1995b) Retroviral retargeting by envelopes expressing an N-terminal binding domain. *J. Virol. 69*, 6314−6322.

(48) S. Valesia-Wittman, F. J. Morling, B. H. K. Nilson, Y. Takeuchi, S. J. Russell, and F.-L. Cosset (1996) Improvement of retroviral retargeting by using amino acid spacers between an additional binding domain and the N-terminus of Moloney leukemia virus SU. *J. Virol. 70*, 2059−2064.

(49) M. Marin, D. Noel, S. Valesia-Wittman, F. Brockly, M. Etienne-Julan, S. Russell, F.-L. Cosset, and M. Piechaczyk (1996) Targeted infection of human cells via Major Histocompatibility Complex Class I molecules by Moloney murine leukemia virus-derived viruses displaying single-chain antibody fragment-envelope fusion proteins. *J. Virol. 70*, 2957−2962.

(50) B. H. Nilson, F. J. Morling, L.-F. Cosset, and S. J. Russell (1996) Targeting of retroviral vectors through protease-substrate interactions. *Gene Ther. 3*, 280−286.

(51) D. Kabat (1995) Targeting retroviral vectors to specific cells. *Science 269*, 417.

(52) V. N. Krasnykh, G. V. Mikheeva, J. T. Douglas, and D. T. Curiel (1996) Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J. Virol. 70*, 6839−6846.

(53) S. I. Michael, J. S. Hong, D. T. Curiel, and J. A. Engler (1995) Addition of a short peptide to the adenovirus fiber protein. *Gene Ther. 2*, 660−668.

(54) J. S. Hong and J. A. Engler (1996) Domains required for assembly of adenovirus type 2 fiber trimers. *J. Virol. 70*, 7071−7078.

(55) B. A. Huber, C. A. Richards, and T. A. Krenitsky (1991) Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy. *Proc. Natl. Acad. Sci. U.S.A. 88*, 8039−8043.

(56) A. Ido, K. Nakata, Y. Kato, K. Nakao, K. Murata, M. Fujita, N. Ishii, T. Tamaoki, H. Shiku, and S. Nagataki (1995) Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase under the control of human α-fetoprotein gene promoter. *Cancer Res. 55*, 3105−3109.

(57) N. K. Wills, W. M. Huang, M. P. Harris, T. Machemer, D. C. Maneval, and R. J. Gregory (1995) Gene-therapy for hepatocellular carcinoma; chemosensitivity conferred by adenovirus mediated transfer of the HSV-1 thymidine kinase gene. *Cancer Gene Ther. 2*, 191−197.

(58) S. Kaneko, P. Hallenbeck, T. Kotani, H. Nabayashi, G. McGarrity, T. Tamaoki, W. F. Anderson, and Y. L. Chiang (1995) Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res. 55*, 5283−5287.

(59) H. Su, J. C. Chiang, S. M. Xu, and Y. W. Kan (1996) Selective killing of AFP-positive cells by adeno-associated virus transfer of the herpes simplex virus thymidine kinase gene. *Hum. Gene Ther. 7*, 463−470.

(60) R. I. Garver Jr., K. T. Goldsmith, B. Rodu, P. C. Hu, and D. T. Curiel (1994) Strategy for achieving selective killing of carcinomas. *Gene Ther. 1*, 46−50.

(61) S. C. Ko, J. Cheong, C. Kao, A. Gotoh, T. Shirakawa, R. A. Sikes, G. Karsenty, and L. K. W. Chung (1996) Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res. 56*, 4614−4619.

(62) T. Kumagai, Y. Tanio, T. Osaki, S. Hosoe, Y. Tachibana, K. Veno, T. Kijima, T. Horai, and T. Kishimoto (1996) Eradication of Myc-overexpressing small cell lung cancer cells transfected with herpes simplex virus thymidine kinase gene containing Myc-Max response elements. *Cancer Res. 56*, 354−358.

(63) T. Osaki, Y. Tanio, I. Tachibana, S. Hosoe, T. Kumagai, I. Kawase, S. Oikawa, and T. Kishimoto (1994) Gene therapy for carcinoembryonic antigen-producing cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene. *Cancer Res. 54*, 5258−5261.

(64) J. M. DiMaio, B. M. Clary, D. F. Via, E. Coveney, T. N. Pappas, and H. K. Lyerly (1994) Directed enzyme pro-drug gene therapy for pancreatic cancer in vivo. *Surgery 116*, 205−213.

(65) T. Tanaka, F. Kanai, S. Okabe, Y. Yoshida, H. Wakimoto, H. Hamada, Y. Shiratori, K. Lan, M. Ishitobi, and M. Omata (1996) Adenovirus-mediated prodrug gene therapy for carcinoembryonic antigen producing human gastric carcinoma cells in vitro. *Cancer Res. 56*, 1341−1345.

(66) C. A. Richards, E. A. Austin, and B. E. Huber (1995) Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. *Hum. Gene Ther. 6*, 881−893.

(67) J. M. Bosher, T. Williams, and H. C. Hurst (1995) The developmentally regulated transcription factor AP-2 is involved in c-erbB2 overexpression in human mammary carcinoma. *Proc. Natl. Acad. Sci. U.S.A. 92*, 744−747.

(68) D. P. Hollywood and H. C. Hurst (1993) A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-erbB-2 in mammary tumour lines. *EMBO J. 12*, 2369−2375.

(69) J. D. Harris, A. A. Gutierrez, H. C. Hurst, K. Sikora, and N. R. Lemoine (1994) Gene therapy for cancer using tumor-specific prodrug activation. *Gene Ther. 1*, 170−175.

(70) Y. Manome, M. Abe, M. F. Hagen, H. A. Fine, and D. W. Kufe (1994) Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer. *Cancer Res. 54*, 5408−5413.

(71) L. Chen, D. Chen, Y. Manome, Y. Dong, H. A. Fine, and D. W. Kufe (1995) Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses controling the DF3/MUCI promoter. *J. Clin. Invest. 96*, 2775−2782.

(72) F. L. Moolten and J. M. Wells (1990) Curability of tumors bearing Herpes virus thymidine kinase genes transferred by retroviral vectors. *J. Natl. Cancer Inst. 82*, 297−300.

(73) R. G. Vile and I. R. Hart (1993a) In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer Res. 53*, 962−967.

(74) R. G. Vile and I. R. Hart (1993b) Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. *Cancer Res. 53*, 3860−3864.

(75) B. W. Hughes, A. H. Wells, Z. Bebok, V. K. Gadi, R. I. Garver Jr., W. B. Parker, and E. J. Sorscher (1995) Bystander killing of melanoma cells using the human tyrosinase promoter to express the E. Coli purine nucleoside phosphorylase gene. *Cancer Res. 55*, 3339−3345.

(76) I. R. Hart (1996) Tissue-specific promoters in targeting systemically delivered gene therapy. *Semin. Oncol. 23*, 154−158.

(77) M. E. Black, T. G. Newcomb, H. M. Wilson, and L. A. Loeb (1996) Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. *Proc. Natl. Acad. Sci. U.S.A. 93*, 3525−3529.

(78) R. Vile, J. A. Nelson, S. Castledon, H. Chang, and I. Hart (1994) Systemic gene therapy of murine melanoma using tissue-specific expression of HSV-TK gene involves an immune response. *Cancer Res. 54*, 6228−6234.

(79) S. Gagandeep, R. Brew, B. Green, S. E. Christmas, D. Klatzmann, G. J. Poston, and A. R. Kinsella (1996) Prodrug-activated gene therapy: Involvement of an immunological component in the "bystander effect". *Gene Ther. 3*, 83−88.

(80) M. Caruso, Y. Panis, S. Gagandeep, D. Houssin, J. L. Salzmann, and D. Klatzmann (1993) Regression of established macroscopic liver metastases after *in situ* transduction of a suicide gene. *Proc. Natl. Acad. Sci. U.S.A. 90*, 7024−7028.

(81) D. R. Averett, G. A. Kozalka, J. A. Fyfe, G. B. Roberts, D. J. M. Purifoy, and T. A. Krenitsky (1991) 6-methoxypurine arabinoside as a selective and potent inhibitor of varicella-zoster virus. *Antimicrob. Agents Chemother. 35*, 851−857.

(82) G. B. Elion (1980) The chemotherapeutic exploitation of virus-specified enzymes. *Adv. Enzymes Regul. 18*, 53−66.

(83) H. S. Allaudeen, J. Descamps, R. K. Sehgal, and J. J. Fox (1982) Selective inhibition of DNA replication in herpes simplex virus infected cells by 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine. *J. Biol. Chem. 257*, 11879−11882.

(84) H. Wallace, A. R. Clarke, D. J. Harrison, M. L. Hooper, and J. O. Bishop (1996) Ganciclovir-induced ablation of non-proliferating thyrocytes expressing herpes virus thymidine kinase occurs by p53-independent apoptosis. *Oncogene 13*, 55−61.

(85) J. G. Tjuvajev, R. Finn, K. Watanabe, R. Joshi, T. Oku, J. Kennedy, B. Beattie, J. Koutcher, S. Larson, and R. G. Blasberg (1996) Noninvasive imaging of herpes simplex virus thymidine kinase gene transfer and expression: A potential method for monitoring clinical gene therapy. *Cancer Res. 56*, 4087−4095.

(86) M. G. Sacco, L. Mangiovini, A. Villa, P. Macchi, O. Barbieri, M. C. Sacchi, E. Monteggia, V. Fasolo, P. Vezzoni, and L. Clerici (1995) Local regression of breast tumors following intramammary ganciclovir administration in double transgenic mice expressing neu oncogene and herpes simplex virus thymidine kinase. *Gene Ther. 2*, 493−497.

(87) (1995) Clinical protocol list. *Cancer Gene Ther. 2*, 225−234.

(88) F. L. Moolten (1986) Tumor chemosensitivity conferred by inserted Herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy. *Cancer Res. 46*, 5276−5281.

(89) F. L. Moolten, B. K. Vonderhaar, and P. J. Mroz (1996) Transduction of the herpes thymidine kinase gene into premalignant murine mammary epithelial cells renders subsequent breast cancers responsive to ganciclovir therapy. *Hum. Gene Ther. 7*, 1197−1204.

(90) E. Borelli, R. Heyman, M. Hsi, and R. M. Evans (1988) Targeting of an inducible toxic phenotype in animal cell. *Proc. Natl. Acad. Sci. U.S.A. 85*, 7572−7576.

(91) D. K. Hoganson, R. K. Batra, J. C. Olsen, and R. C. Boucher (1996) Comparison of the effect of three different toxin genes and their levels of expression on cell growth and bystander effect in lung adenocarcinoma. *Cancer Res. 56*, 1315−1323.

(92) S. Kuriyama, T. Nakatani, K. Masui, T. Sakamoto, K. Tominaga, M. Yoshikawa, H. Fukui, K. Ikenaka, and T. Tsujii (1996) Evaluation of prodrugs ability to induce effective ablation of cells transduced with viral thymidine kinase gene. *Anticancer Res. 16*, 2623−2628.

(93) K. W. Culver, Z. Ram, S. Wallbridge, H. E. H. Oldfield, and M. R. Blaese (1992) In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science 256*, 1550−1552.

(94) D. Barba, J. Hardin, J. Ray, and F. H. Gage (1993) Thymidine kinase-mediated killing of rat brain tumors. *J. Neurosurg. 79*, 729−735.

(95) D. Barba, J. Hardin, M. Sadelain, and F. H. Gage (1994) Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. *Proc. Natl. Acad. Sci. U.S.A. 91*, 4348−4352.

(96) S. H. Chen, H. D. Shine, J. C. Goodman, R. G. Grossman, and S. L. C. Woo (1994) Gene therapy for brain tumors: Regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc. Natl. Acad. Sci. U.S.A. 91*, 3054−3057.

(97) Z. Ram, K. W. Culver, S. Walbridge, R. M. Blaese, and E. H. Oldfield (1993) In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res. 53*, 83−88.

(98) S. J. Tapscott, A. D. Miller, J. M. Olson et al. (1994) Gene therapy of 9L rat gliosarcoma tumors by transduction with selectable genes does not require drug selection. *Proc. Natl. Acad. Sci. U.S.A. 91*, 8185−8189.

(99) N. G. Rainov, C. M. Kramm, K. Aboody-Guterman, M. Chase, K. Ueki, D. N. Loius, G. R. Harsh IV, E. A. Chiocca, and X. O. Breakefield (1996) Retrovirus-mediated gene therapy of experimental brain neoplasms using the herpes simplex virus-thymidine kinase/ganciclovir paradigm. *Cancer Gene Ther. 3*, 99−106.

(100) A. Maron, T. Gustin, A. Le Roux, I. Mottet, J. F. Dedieu, J. P. Brion, R. Demeure, M. Perricaudet, and J. N. Octave (1996) Gene therapy of rat C6 glioma using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene: long time follow-up by magnetic resonance imaging. *Gene Ther. 3*, 315−322.

(101) V. Cool, B. Pirotte, C. Gerard, J. L. Dargent, N. Baudson, M. Levivier, S. Goldman, J. Hildebrandt, J. Brotchi, and T. Velu (1996) Curative potential of herpes simplex virus thymidine kinase gene transfer in rats with 9L gliosarcoma. *Hum. Gene Ther. 7*, 627−635.

(102) A. J. P. E. Vincent, R. Vogels, G. V. Someren, M. C. Esandi, J. L. Noteboom, C. J. J. Avezaat, C. Vecht, D. W. V. Bekkum, D. Valerio, A. Bout, and P. M. Hoogerbrugge (1996) Herpes simplex virus thymidine kinase gene therapy for rat malignant brain tumors. *Hum. Gene Ther. 7*, 197−205.

(103) M. E. Rosenfeld, M. Wang, G. P. Siegal, R. D. Alvarez, G. Mikheeva, V. Krasnykh, and D. T. Curiel (1996) Adenoviral-mediated delivery of herpes simplex virus thymidine kinase results in tumor reduction and prolonged survival in SCID mouse model of human ovarian carcinoma. *J. Mol. Med. 74*, 455−462.

(104) D. Yee, S. E. McGuire, N. Brunner, T. W. Kozelsky, D. C. Allred, S. H. Chen, and S. L. C. Woo (1996) Adenovirus-mediated gene transfer of herpes simplex virus thymidine kinase in an ascites model of human breast cancer. *Hum. Gene Ther. 7*, 1251−1257.

(105) J. A. Eastham, S. H. Chen, I. Sehgal, G. Yang, T. L. Timme, S. J. Hall, S. L. C. Woo, and T. C. Thompson (1996) Prostate cancer gene therapy: Herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. *Hum. Gene Ther. 7*, 515−523.

(106) M. MacCoss and M. J. Robins (1990) In *Chemistry of Antitumour Agents* (D. E. V. Wilman, Ed.) pp 261−298, Blackie & Son Ltd., Chapman & Hall, London, New York.

(107) D. S. Martin (1987) In *Metabolism and Action of Anticancer Drugs* (G. Powis and R. A. Prough, Eds.) pp 91−140, Taylor & Francis, London.

(108) C. A. Mullen, M. Kilstrup, and R. M. Blaese (1992) Transfer of the bacterial gene for cytosine deaminase to a mammalian cells confers lethal sensitivity to 5-fluorocytosine: A negative selection system. *Proc. Natl. Acad. Sci. U.S.A. 89*, 33−37.

(109) U. Haberkorn, F. Oberdorfer, J. Gebert, I. Morr, K. Haack, K. Weber, M. Lindauer, G. Van Kaik, and H. K. Schackert (1996) Monitoring gene therapy with cytosine deaminase: In vitro studies using tritiated-5-fluorocytosine. *J. Nucl. Med. 37*, 87−94.

(110) B. E. Huber, E. A. Austin, V. C. Good, V. C. Knick, S. Tibbels, and C. A. Richards (1993) In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res. 53*, 4619−4626.

(111) B. E. Huber, E. A. Austin, C. A. Richards, S. T. Davis, and S. S. Good (1994) Metabolism of 5-fluorocytidine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: Significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc. Natl. Acad. Sci. U.S.A. 91*, 8302−8306.

(112) A. Ohwada, E. A. Hirschowitz, and R. G. Crystal (1996) Regional delivery of an adenovirus vector containing the Escherichia coli cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum. Gene Ther. 7*, 1567−1576.

(113) N. E. Sladek (1987) In *Metabolism and Action of Anticancer drugs* (G. Powis and R. A. Prough, Eds.) pp 48−89, Taylor & Francis, London.

(114) L. Bielicki, G. Voelcker, and H. J. Hohorst (1984) Activated cyclophosphamide: an enzyme-mechanism-based suicide inactivator of DNA polymerase/3′,5′ exonuclease. *J. Cancer Res. Clin. Oncol. 107*, 195−198.

(115) T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster, A. M. Gilsenan, and M. Jarman (1974) Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. *Biochem. Pharmacol. 23*, 115−129.

(116) T. K. H. Chang, G. F. Weber, C. L. Crespi, and D. J. Waxman (1993) Differential activation of cyclophosphamide and ifosphamide by cytochromes P450 2B and 3A in human liver microsomes. *Cancer Res. 53*, 5629−5637.

(117) M. X. Wei, T. Tamiya, M. Chase, E. J. Boviatsis, T. K. Chang, N. W. Kowall, F. H. Hochberg, D. J. Waxman, X. O. Breakefield, and E. A. Chiocca (1994) Experimental tumor therapy in mice using the cyclophosphamide-activating cytochrome P450−2B1 gene. *Hum. Gene Ther. 5*, 969−978.

(118) J. Halpern, J. Y. Jaw, L. J. Cornfield, C. Balfour, and E. Mash (1989) Selective inactivation of rat liver cytochromes P450 by 21-chlorinated steroids. *Drug Metab. Disp. 17*, 26−31.

(119) M. A. Correia, K. Yao, S. A. Wrighton, D. J. Waxman, and A. E. Rettie (1992) Differential apoprotein loss of rat liver cytochromes P450 after their inactivation by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine: a case for distinct proteolytic mechanism. *Arch. Biochem. Biophys. 294*, 493−503.

(120) Y. Manome, P. Y. Wen, L. Chen, T. Tanaka, Y. Dong, M. Yamazoe, A. Hirshowitz, D. W. Kufe, and H. A. Fine (1996) Gene therapy for malignant gliomas using replication incompetent retroviral and adenoviral vectors encoding the cytochrome P-450 2B1 gene together with cyclophosphamide. *Gene Ther. 3*, 513−520.

(121) L. Chen, D. J. Waxman, D. Chen, and D. C. Kufe (1996) Sensitization of human breast cancer cells to cyclophosphamide and ifosfamide by transfer of a liver cytochrome P-450 gene. *Cancer Res. 56*, 1331−1340.

(122) T. P. Zimmerman, N. B. Gersten, A. F. Ross, and R. P. Miech (1971) Adenine as substrate for purine nucleoside phosphorylase. *Can. J. Biochem. 49*, 1050−1054.

(123) E. J. Sorscher, S. Peng, Z. Bebok, P. W. Allan, L. L. Bennett Jr., and W. B. Parker (1994) Tumor cell bystander killing in colonic carcinoma utilizing the *Escherichia coli* deo-D gene to generate toxic purines. *Gene Ther. 1*, 233−238.

(124) A. V. Patterson, H. Zhang, A. Moghaddam, R. Bicknell, D. C. Talbot, I. J. Stratford, and A. L. Harris (1995) Increased sensitivity to the prodrug 5′-deoxy-5-fluorouridine and modulation of 5-fluoro-2′-deoxyuridine sensitivity in MCF-7 cells transfected with thymidine phosphorylase. *Br. J. Cancer 72*, 669−675.

(125) D. M. Hapke, A. P. A. Stegmann, and B. S. Mitchell (1996) Retroviral transfer of deoxycytidine kinase into tumor cell lines enhances nucleoside toxicity. *Cancer Res. 56*, 2343−2347.

(126) Y. Manome, P. Y. Wen, Y. Dong, T. Tanaka, B. S. Mitchell, D. W. Kufe, and H. A. Fine (1996) Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside in vitro and in vivo. *Nat. Med. 2*, 567−573.

(127) R. F. Sherwood, R. G. Melton, S. M. Alwan, and P. Hughes (1985) Purification and properties of carboxypeptidase G2 *Pseudonomas sp* strain RS-16. *Eur. J. Biochem. 148*, 447−453.

(128) C. J. Springer, P. Antoniw, K. D. Bagshawe, F. Searle, G. M. F. Bisset, and M. Jarman (1990) Novel prodrugs which are activated to cytotoxic alkylating agents by carboxypeptidase G2. *J. Med. Chem. 33*, 677−681.

(129) R. Dowell, C. J. Springer, D. H. Davies, E. M. Hadley, P. J. Burke, F. T. Boyle, R. G. Melton, T. A. Connors, D. C. Blakey, and A. B. Mauger (1996) New mustard prodrugs for antibody-directed enzyme prodrug therapy: Alternative for the amide link. *J. Med. Chem. 39*, 1100−1105.

(130) C. J. Springer, R. Dowell, P. J. Burke, E. Hadley, D. H. Davies, D. C. Blakey, R. G. Melton, and I. Niculescu-Duvaz (1995) Optimization of alkylating agent prodrugs derived from phenol and aniline mustards: A new clinical candidate prodrug (ZD2767) for antibody-directed enzyme prodrug therapy (ADEPT). *J. Med. Chem. 38*, 5051−5065.

(131) A. C. Khan and W. C. J. Ross (1967) Tumour growth inhibitory nitrophenylaziridines and related compounds: structure activity relationships. *Chem. Biol. Interact. 1*, 27−47.

(132) R. J. Knox, M. P. Boland, F. Friedlos, B. Coles, C. Southan, and J. J. Roberts (1988) The nitroreductase enzyme in Walker cells that activates 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2). *Biochem. Pharmacol. 44*, 2297−2301.

(133) R. J. Knox, F. Friedlos, R. F. Sherwood, R. G. Melton, and G. M. Anlezark (1992) The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954). II. A comparison of an Escherichia coli nitroreductase and Walker DT-diaphorase. *Biochem. Pharmacol. 44*, 2297−2301.

(134) M. P. Boland, R. J. Knox, and J. J. Roberts (1991) The differences in kinetics of rat and human DT-diaphorase result in in a differential sensitivity of cells lines to CB 1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide. *Biochem. Pharmacol. 41*, 867−875.

(135) R. C. Mulligan and P. Berg (1981) Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. U.S.A. 78*, 22072−22076.

(136) T. Tamiya, Y. Ono, M. X. Wei, P. J. Mroz, F. L. Moolten, and E. A. Chiocca (1996) *Escherichia coli* gpt gene sensitizes rat glioma cells to killing by 6-thioxanthine or 6-thioguanine. *Cancer Gene Ther. 3*, 155−162.

(137) A. R. Kinsella, D. Smith, and M. Pickard (1997) Resistance to chemotherapeutic antimetabolites: a function of salvage pathway involvement and cellular response to DNA damage. *Br. J. Cancer 75*, 935−945.

(138) L. Chen and D. J. Waxman (1995) Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P-450 gene transfer: Development of a combined chemotherapy/cancer gene therapy strategy. *Cancer Res. 55*, 581−589.

(139) P. T. Golumbek, F. M. Hamzeh, E. M. Jaffee, H. Levitsky, P. S. Lietman, and D. M. Pardoll (1992) Herpes simplex-1 virus thymidine kinase gene is unable to completely eliminate live, nonimmunogenic tumour cell vaccines. *J. Immunother. 12*, 224−230.

(140) B. A. Teicher and E. Frei III (1988) Development of alkylating agent resistant human tumor cell lines. *Cancer Chemother. Pharmacol. 21*, 292−298.

(141) E. Frei, B. A. Teicher, and S. A. Holden (1988) Preclinical studies and and clinical correlation of the effect of alkylating drugs. *Cancer Res. 48*, 6417.

(142) S. M. Freeman, C. N. Abboud, K. A. Whartenby, C. H. Packman, D. S. Koeplin, F. S. Moolten, and G. N. Abraham (1993) The "bystander effect": Tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res. 53*, 5274−5283.

(143) Q. T. Trinh, E. A. Austin, D. M. Murray, V. C. Knick, and B. E. Huber (1995) Enzyme/prodrug gene therapy: Comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug system in a human colorectal cacinoma cell line. *Cancer Res. 55*, 4808−4812.

(144) G. Goldberg and J. S. Bertram (1994) Correspondence re: Z. Ram et al. In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res. 53*, 83−88; (1993) *Cancer Res. 54*, 3947−3948.

(145) S. F. Dagher, S. E. Conrad, E. A. Werner, and R. J. Patterson (1992) Phenotypic conversion of TK-defficient cells following electroporationof functional TK enzyme. *Exp. Cell Res. 198*, 36−42.

(146) W. L. Bi, L. M. Parysek, R. Warnick, and P. J. Stambrook (1993) *In vitro* evidence that metabolic cooperation is responsible for the bystander effect observed with HSV *tk* retroviral gene therapy. *Hum. Gene Ther. 4*, 725−731.

(147) J. Fick, F. G. Barker, P. Dazin, E. M. Westphale, E. C. Beyer, and M. A. Israel (1995) The extent of heterocellular communication mediated gap junctions is predictive of bystander tumor cytotoxicity *in vitro*. *Proc. Natl. Acad. Sci. U.S.A. 92*, 11071−11075.

(148) S. M. Freeman, R. Ramesh, M. Sastri, A. Munnshi, A. K. Jensen, and A. J. Marrogi (1995) The role of cytokines in mediating the bystander effect using HSV-TK xenogeneic cells. *Cancer Lett. 92*, 167−174.

(149) W. Hamel, L. Magnelli, V. P. Chiarugi, and M. A. Israel (1996) Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. *Cancer Res. 56*, 2697−2702.

(150) R. Ramesh, A. J. Marrogi, A. Munshi, C. N. Abboud, and S. M. Freeman (1996) In vivo analysis of the "bystander effect": A cytokine cascade. *Exp. Hematol. 24*, 829−838.

(151) J. Pavlovic, M. Nawrath, R. Tu, T. Heinicke, and K. Moelling (1996) Anti-tumor immunity is involved in the thymidine kinase-mediated killing of tumors induced by activated Ki-ras (G12V). *Gene Ther. 3*, 635−643.

(152) R. Vile, N. Miller, Y. Chernayovsky, and I. Hart (1994) 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. 1*, 307−316.

(153) S. H. Chen, X. L. Li Chen, Y. Wang, K. Kosai, M. J. Finegold, S. S. Rich, and S. L. C. Woo (1995) Combination gene therapy for liver metastasis of colon carcinoma in vivo. *Proc. Natl. Acad. Sci. U.S.A. 92*, 2577−2581.

(154) S. H. Chen, K. Kosai, B. Xu, K. Pham-Nguyen, C. Contant, M. J. Finegold, and S. L. C. Woo (1996) Combination suicide and cytokine gene therapy for hepatic metastases of colon carcinoma: Sustained antitumor immunity prolongs animal survival. *Cancer Res. 56*, 3758−3762.

(155) B. W. O'Malley, K. A. Cope, S. H. Chen, D. Li, M. R. Schwartz, and S. L. C. Woo (1996) Combination gene therapy for oral cancer in a murine model. *Cancer Res. 56*, 1737−1741.

BC970116T