An investigation of enzyme / prodrug systems for cancer gene therapy

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To Lin and my family

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List of abbreviations

ADEPT	antibody directed enzyme / prodrug therapy
AFP	α-foetoprotein
AS	antisense
β-gal	β-galactosidase
BHK	baby hamster kidney
CarB-E	CarB-E cadherin transfected cells
CB1954	5-arizidin-1-yl-2,4 dinitrobenzamide
cd	cytosine deaminase
CEA	carcinoembryonic antigen
cfu	colony forming units
CMV	Cytomegalovirus
CpG2	carboxypeptidase G2
Cx	connexin
DFUR	5'-deoxy-5-fluorouridine
DNA	deoxyribonucleic acid
FC	fluorocytosine
FCS	foetal calf serum
FU	fluorouracil
FUdR	5-fluoro-2'-deoxyuridine
FUR	5-fluorouridine
GCV	ganciclovir
GCV-MP	ganciclovir - monophosphate
GCV-TP	ganciclovir - triphosphate
GVHD	graft versus host disease
GJIC	gap junctional intercellular communication
GCSF	granulocyte colony stimulating factor
GMCSF	granulocyte-macrophage colony stimulating factor
HPRT	hypoxanthine phosphoribosyl transferase
hr	hour(s)
IL	interleukin
IFN	interferon
i.p.	intraperitoneal
IRES	internal ribosome entry site
i.v.	intravenous
MHC	major histocompatability class
min	minute(s)
MOI	multiplicity of infection
MP	methylpurine
MP-d	methylpurine-2'deoxyribonucleoside
ntr	nitroreductase
OR	Objective Response
p170	multiple drug resistance protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PNP	purine nucleoside phosphorylase

RNA	ribonucleic acid
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TG	thioguanine
TNF	tumour necrosis factor
TIL	tumour infiltrating lymphocyte
tk	Herpes Simplex virus thymidine kinase
tp	human thymidine phosphorylase
tyr	tyrosinase
TPA	12-O-tetradecanyol phorbol-13-acetate
vpc	vector producer cell
x-gal	5-Bromo-4-Chloro-3-Indyol β-D-Galactopyranoside

Publications

The results presented in this thesis have been published, in part, in the following journal.

Denning C; Pitts J,D: Bystander effects of different enzyme / prodrug systems for cancer gene therapy depend on different pathways for intercellular transfer of toxic metabolites, a factor that will govern choice of appropriate regimes. Human Gene Therapy. 8:1825-1835, 1997

Declaration

All the work in this thesis was my own. Some services were provided by the Beatson institute. These included, cryosectioning and x-gal staining of tumour samples (Brenda McGuire) and sequence analysis of prepared DNA samples (Robert Mcfarlane). In addition, *in vivo* experiments was assisted by the staff in Central Services. The work submitted was undertaken under the supervision and guidance of Dr. John Pitts. No part of this work has been submitted for consideration for any other degree or award.

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Abstract

Chemotherapy, radiotherapy and surgery, the current treatments for cancer, cure only fifty percent of patients. Gene therapy, where therapeutic genes are transferred directly to the tumour, offers a new form of cancer treatment. However, gene transfer techniques that are currently available can only achieve gene expression in a small proportion of the tumour cells, and this may hinder many gene therapy approaches. It may therefore be important to select genes for cancer therapy that offer the potential for a significant bystander effect, where cells not expressing the therapeutic gene are killed, as well as targeted cells.

At present there appear to be two strategies for cancer gene therapy that achieve bystander killing, and hence require targeted expression in only a proportion of the cells. The first depends on stimulation of the immune system by, for example, expression of specific cytokines that increase proliferation of tumoricidal cells, while the second type of bystander effect is associated with enzyme / prodrug systems, which are the focus of the work described in this thesis.

The mechanisms and efficacies of the Herpes Simplex virus thymidine kinase / ganciclovir (tk / GCV), human thymidine phosphorylase / 5'-deoxy-5-fluorouridine (tp / DFUR) and E.coli cytosine deaminase / 5-fluorocytosine (cd / FC) enzyme / prodrug systems have been examined. In culture, each enzyme / prodrug combination induced cell death in tumour cells expressing the suicide gene and, in mixed cultures of cells that do or do not express the gene, death occurred in both cell types, indicative of a bystander effect.

With the tk / GCV system, in human and rodent cell lines, a correlation has been demonstrated between the bystander effect and gap junctional intercellular communication (GJIC). When cultures lack GJIC, no bystander effect was observed, but only a low level of

communication was required to generate extensive bystander killing. Increased enzyme activity, on the other hand, improved the bystander effect, suggesting that high levels of tk expression will be important. Additionally, it has been shown that transfer of toxic metabolites from tk^+ to tk^- cells occurs within two hr of GCV application, before degenerative events were detected, indicating that apoptosis is the result, not the cause, of the tk / GCV bystander effect.

Three of the cell lines used to investigate the tk / GCV system in culture were also used to examine the bystander effect in experimental tumours in nude mice. A correlation between the bystander effect in culture and *in vivo* was demonstrated in two of the cell lines but not the third, which suggests that other factors are important *in vivo*.

In the tp / DFUR and cd / FC systems, bystander killing was due to transfer of toxic metabolites through the medium, and did not depend on GJIC or cell-cell contact. Bystander killing seen with these systems was weaker than that seen in the tk / GCV system.

Combining the tk / GCV system with the tp / DFUR or cd / FC systems in culture was able to produce more extensive bystander killing than either system separately. The improvement was also observed using a cdtk fusion protein.

The results in this thesis suggest that the choice of enzyme / prodrug therapy for cancer will be determined by the characteristics of each tumour. In addition they suggest the use of fusion proteins, to combine therapeutic proteins, warrants further investigation.

Chapter 1. Review of the literature

1.1. Current success of cancer treatment

Cancer is a disease associated with morbidity and mortality. In the United States of America over one million individuals will develop cancer this year but only fifty percent will survive. Cancer is the leading cause of death in females aged 35 to 74, and the second leading cause of death in both sexes (22.5% total deaths in USA based on 1987 figures; Blaese, 1997; Boring et al, 1991). Frequency of certain cancers is rising, for example, the incidence of lung cancer has increased by 5 fold in females during the last 30 years (Boring et al, 1991) and it has been suggested that by the year 2000 it is likely that over one percent (in USA, approximately 4 million) of individuals will develop cutaneous melanoma (Bonnekoh et al, 1996).

Worldwide, over 1.6 million individuals develop hepatocellular carcinoma each year and 80% die (Willis et al, 1995). In addition, colorectal carcinomas lead to the death of 175,000 people, annually in USA, UK, Japan, France, Germany, Italy and Spain (Huber et al, 1995).

In Scotland, mortality rates due to cancer were 2nd and 7th highest (female and male, respectively) of 48 countries studied from 1984 to 1986 (Boring et al, 1991).

1.2. Current cancer treatments

Routine treatment for cancer involves surgery, radio- and chemo-therapy. All of these treatments can be effective but, as described above, 50% of tumours remain incurable. Although surgery removes the cancerous cells, residual cells may repopulate the tumour or

lead to metastatic deposits. Tumours that are inoperable must be treated with radio- or chemo-therapy, which exploit the modest differences between non-neoplastic and neoplastic cells. For example, vinca alkaloids, such as vinblastine and vincristine, are cell cycle dependent, antimitotic chemotherapeutic agents that bind to the cytoskeletal protein tubulin, inducing microtubule depolymerisation. This prevents the formation of the mitotic spindle and rapidly dividing cells are most susceptible to killing. 5-fluorouracil (FU) is a chemotherapeutic agent that is converted by cellular enzymes to cytotoxic metabolites that interfere with RNA and DNA synthesis. Thymidine phosphorylase (tp) is of one of the enzymes involved in the conversion and, in certain tumours, such as colorectal carcinoma, the level of tp is increased relative to normal tissue (Birnie et al, 1963; Sumizawa et al, 1993), making some of these tumours suitable for FU treatment.

A common feature of chemotherapeutic agents is a narrow therapeutic index, therefore systemic toxicity is dose limiting. For example, vincristine can result in neurotoxicity, while vinblastine can result in leukopenia, and FU often causes leukopenia and gastric ulceration (Ratain and Vogelzang et al, 1986; Au et al, 1982). Although systemic toxicity can be reduced by regional drug delivery, for example, melanomas or hepatic carcinomas can be isolated by limbic perfusion or hepatic vascular perfusion, respectively, these procedures require surgery (Anderson et al, 1992). Regional delivery allows higher doses of drug to be administered but many solid tumours require a dose that still cannot be achieved (review, Connors and Knox, 1995). In addition, tumour cells can become resistant to chemotherapeutic agents through several mechanisms which include expression of the multiple drug resistance protein (p170), improvement in DNA repair mechanisms and alteration of the composition of the plasma membrane lipid composition (review, Vendrik et al, 1992). It is evident that to increase the cure rate of cancer, new therapies are required and gene therapy may provide an additional treatment which can complement existing regimes.

1.3. Gene therapy for cancer

Gene therapy was suggested as an additional means of controlling disease in the 1970's by Friedman. Currently there are a number of approaches that are being investigated for cancer gene therapy (see figure 1.1).

1.4. Targeting of non cancer cells

1.4.1. Gene transfer to lymphocytes

The first studies that investigated transfer of foreign genes to humans, involved introduction of a bacterial gene (neo) to lymphocytes, primarily to assess the safety of gene transfer.

In these studies, tumour infiltrating lymphocytes (TILs) were isolated from metastatic melanoma deposits, then grown in culture (*ex vivo*) and cell number expanded. Prior to re-infusion of the expanded population of TILs, the neo gene was introduced to these cells by retroviral transduction. The recombinant genome of the virus integrates into the genome of the TIL, therefore the persistence of these cells could be monitored (by PCR and Southern blot analysis for the neo gene) both in the circulation and in the tumours of patients. TILs could be detected for up to 2 months, in some tumours, and in ≈30% of cases investigated reinfusion of increased numbers TILs led to regression of tumour deposits (Rosenberg et al, 1990; Rosenberg, 1992). This approach proved safe and has subsequently been extended to generate TILs that secrete interleukin 2 (IL-2) in an attempt to enhance the patients immune response to tumours (Rosenberg et al, 1993).

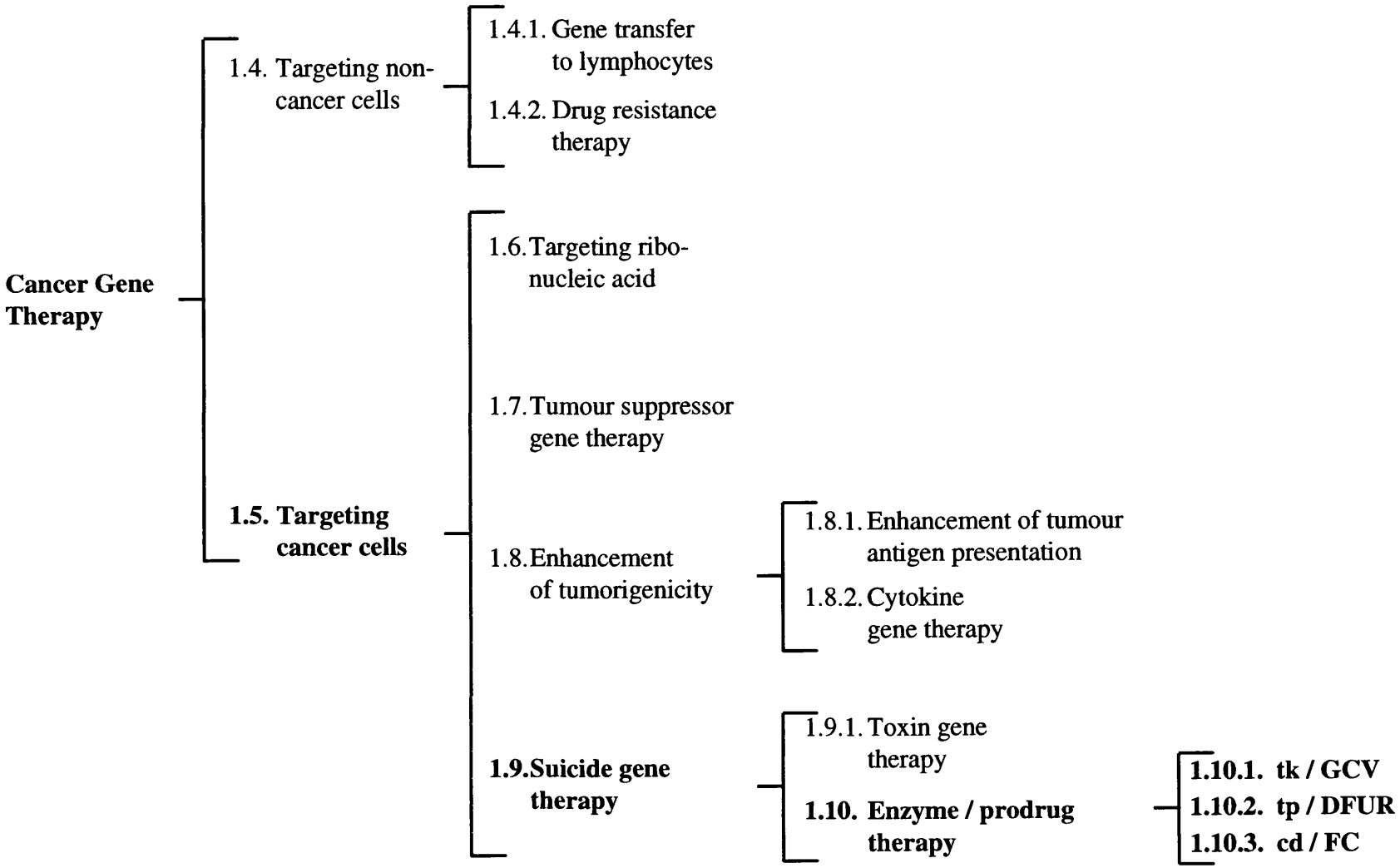


Figure 1.1. Possible methods for genetic intervention in cancer therapy

In another example of *ex vivo* gene transfer, the Herpes Simplex virus thymidine kinase gene (tk; see section 1.10.1.) was transferred, using retroviral transduction, to lymphocytes. Patients with haematological malignancies can be cured by high dose chemotherapy, followed by allogeneic bone marrow transplantation, but graft versus host disease (GVHD) can occur when infused lymphocytes react against host tissue, which can kill the patient. Introduction of the tk gene may allow an aberrant reaction to be attenuated since the prodrug, ganciclovir, can kill cells expressing this gene (Tiberghien et al, 1994; Munshi et al, 1997).

Gene transfer in culture does enable genes to be transferred to a high percentage of cells, but this approach is not always successful (Rosenberg et al, 1990). In some cases it may not be possible to establish primary cultures or to expand the cell number sufficiently for clinical use. In addition, cells may undergo antigenic changes during the culture period and this may lead to cell rejection by the host immune system.

1.4.2. Drug resistance gene therapy

Systemic toxicity associated with chemotherapeutic agents restricts the length and dose of treatment, but it may be possible to reduce the extent of toxicity by protecting stem cells and early progenitor cells. Tumour cell resistance to chemotherapeutic agents may arise from expression of the multiple drug resistance protein, p170. If the gene (*mdr1*) encoding this protein is introduced to stem cells, then they may acquire resistance to certain chemotherapeutic drugs, and it might be possible to increase the dose or the length of drug treatment. This hypothesis was tested by Sugimoto et al (1994; 1995) who transfected cultured fibroblasts with *mdr1* and showed resistance to vincristine and adriamycin.

If this approach was used clinically, it is possible that *mdr1* would also be transferred to tumour cells and drug resistant cancers may arise. To avoid this possibility it would be

desirable to also introduce a conditional suicide gene, such as the tk gene (section 1.10.1), to cells. This approach has been investigated, in culture, and cells that were resistant to chemotherapeutic agents were also sensitive to GCV (Sugimoto et al, 1995). However, these experiments were performed in fibroblasts and whether the approach would also be successful in stem cells will need to be evaluated.

1.5. Targeting tumour cells

Most of the work in cancer gene therapy has involved direct targeting of tumour cells in culture or in experimental models. An overview of some of the methods used for killing tumour cells is provided in figure 1.1. and examples are given below.

1.6. Targeting ribonucleic acid

The mRNA molecules that code for proteins that are over expressed in neoplastic cells may provide targets for antisense therapy. This approach introduces complementary sequences into tumour cells which may disrupt nuclear transport or mark the targeted mRNA for degradation (review, Mercola and Cohen, 1995). For example, the mRNA that codes for the proto-oncogenes, such as bcl-2 and ras, or for the immunosuppressive cytokine TGF-β are potential targets in some cancers.

The efficacy of the antisense approach has been demonstrated by the killing of low grade non Hodgkins lymphoma cells, in culture, by targeting antisense oligodeoxy-ribonucleotides to bcl-2 mRNA (Smith et al, 1997a). Killing by this approach is surprising because the main mechanism of oligonucleotide uptake is by endocytosis and it might be expected that the oligonucleotides would end up, not in the cytoplasm or nucleus, but in the

lysosomal compartment, hence degraded. Studies *in vivo* have shown that experimental, established pancreatic and lung tumours regress when antisense cDNA or genomic sequence to K-ras was introduced by retroviral transduction (Georges et al, 1993; Aoki et al, 1995). Again, this result is not necessarily expected since full length antisense sequences may be stabilised by secondary structure, which could prevent effective alignment with target mRNA.

Ribozymes represent an alternative way to reduce transcript levels. These naturally occurring RNA enzymes can be modified to target, bind and cleave specific mRNA molecules. Ribozymes directed to RNA polymerase 1 mRNA (Voeks et al, 1996) or to telomerase (Kanazawa et al, 1996) mRNA can kill cells in culture, and established, experimental malignant melanomas have been shown to regress when targeted with a ribozyme to H-ras (Ohkawa et al, 1997).

These approaches appear effective, at least in some cases, in culture and in experimental tumours generated from cell lines. However, tumours in humans develop over longer periods of time, and the tumour phenotype arises from several mutations, therefore, correcting single genetic defects clinically, may not be as effective as in model systems.

It may also be anticipated that every cell, or a large proportion of the cells within a tumour, must be targeted with the therapeutic sequences. However, the available data suggest that the level of transduction that can be achieved by retroviruses, in brain tumours, is <0.2% (Ram et al, 1995). If gene transfer is this inefficient in other tumours the use of antisense therapy in humans may not be beneficial.

1.7. Tumour suppressor gene therapy

Tumour progression is often associated with loss or mutation of tumour suppressor genes (anti-oncogenes). The most studied product from this class of genes is p53 which, in

response to DNA damage, can lead to growth arrest allowing subsequent DNA repair or activation of apoptotic pathways (Kastan et al, 1991). In tumours that lack p53 activity, introduction of a functional *p53* gene may be beneficial.

Small, established tumours, derived from a $p53^-$ non small cell lung cancer cell line, have been shown to regress, at least in some cases, when transduced with a retrovirus carrying the *p53* gene (Nguyen et al, 1997; Nielson et al, 1997). Regressions have also been seen in breast and bladder cancer models (Qazilbash et al, 1997).

These results are encouraging, but as with the antisense strategy, correction of a single genetic defect may be insufficient to be of benefit clinically.

1.8. Enhancement of immunogenicity

To develop, tumours must evade immune detection. This may occur through loss of tumour antigens, through loss of antigen presentation machinery or by lack of appropriate stimulatory signals. Introducing components involved in immune stimulation can, in some cases, lead to tumour regression. Furthermore, activation of the immune system can lead to a bystander effect, where cells not expressing the therapeutic gene are killed as well as the targeted cells. Some of the investigations to determine which components of the immune system are important are described below.

1.8.1. Enhancement of tumour antigen presentation

Evasion of immune detection by tumour cells often correlates with reduced levels of antigen presenting molecules (e.g. histocompatability complex; MHC) or co-stimulatory molecules (e.g. B7) which are required for efficient activation of $CD8^+$ cytotoxic lymphocytes

(Rubin et al, 1997; Wendtner et al, 1997). Ma et al (1996) have shown transduction of B16 melanomas in culture with MHC II or B7 abrogates the tumorigenicity of these cells in syngeneic mice. Furthermore, when animals were immunised with these transduced cells, a subsequent rechallenge of B16 parental cells was rejected in some cases, indicative of a bystander effect, in the form of immune memory in this instance.

Loss of tumorigenicity does not occur in all cell lines transduced with MHC II or B7 and it is likely that this can be explained by the correlation between antitumour effect and inherent immunogenicity of the tumour (Chen et al, 1994). However some lines of evidence suggest that enhancing tumour immunogenicity with cytokines, such as interleukin 2 or interleukin 12, may increase the effectiveness of MHC and B7 expression (Ma et al, 1996; Lotze et al, 1997).

Enhancing the immune response with cytokines has been the focus of many reports and details of some of the findings are given in the next section.

1.8.2. Cytokine gene therapy

Early attempts to enhance the immune response to tumours involved intratumoural injection with non specific adjuvants such as *Corynebacterium parvum*. The basis of this approach was that the inflammatory reaction to a foreign protein would increase antigen presentation and cause an infiltration of T-lymphocytes to the vicinity of the tumour. Current efforts to express cytokine genes in tumour cells are an extension of this concept (review, Miller et al, 1994).

Cytokines are small secreted glycoproteins that are required for activation and proliferation of cells of the immune system, such as T-cells, granulocytes and macrophages. Interest has arisen in these proteins because systemic delivery of some (e.g. tumour necrosis

factor-α, TNF-α; interleukin-2, IL-2) has been shown to induce tumour regression of experimental animal tumours. However, the systemic doses that can be achieved in humans are up to 50-fold lower than those in mice, and clinical use of TNF-α or IL-2 has led to systemic toxicity, or even death of patients (Rosenberg, 1992).

Expression of cytokine genes within tumour cells may produce high local concentrations of cytokine that could have an antitumour effect, without systemic toxicity. However, determining the mechanism of action of individual cytokines is often hindered by redundancy, or the events that occur following expression. For example, IL-2 activates B-cells, T-cells, natural killer (NK) cells and macrophages, but these cells can release other cytokines, including TNF-α, interferon-γ (IFNγ) and granulocyte-macrophage colony stimulating factor (GMCSF). In turn, these cytokines may lead to secretion of others. This cascade means that studying the action of a single cytokine is difficult (review, Miller et al, 1994).

Some reports have described the effects of several cytokines in one tumour model (Dranoff et al, 1993; Allione et al, 1994) but as the intrinsic properties of each model varies, so does the action of the cytokine. For example, the tumorigenicity of mammary adenocarcinoma engineered to express IFNγ was reduced in syngeneic mice while expression of IL-6 had no significant antitumour effect (Allione et al, 1994), but in B16 melanoma, the reverse was true (Dranoff et al, 1993). In a separate report, immunisation of syngeneic mice with melanoma cells expressing IL-2 was shown to induce specific, systemic immunity to a subsequent challenge of B16 cells (Ma et al, 1996), but this effect had not been seen previously (Dranoff et al, 1993). These differences may be explained by the observation that in some tumours IL-2 has a dose dependent window, and higher or lower concentrations of the cytokine reduce protection (Cavallo et al, 1992).

The effects of particular cytokines are consistent, between reports, in some animal models. For example, the tumorigenicity of B16 melanoma cells expressing IL-2 is reduced (Dranoff et al, 1993; Allione et al, 1994; Ma et al, 1996) and γ-irradiated B16 cells expressing GMCSF, or live B16 cells expressing IL-2 and GMCSF, generate specific, systemic immunity to parental cell rechallenge, in some cases (Dranoff et al, 1993; Yu et al, 1997).

Producing a systemic response is of particular interest because even when only a fraction of the tumour is transduced with these cytokines, it may lead to complete destruction of local and distant tumour deposits. This bystander effect is absent from animals depleted of $CD4^+$ and $CD8^+$ lymphocytes, suggesting that these cells are essential in the response (Fearon et al, 1990; Dranoff et al, 1993; Lotze et al, 1997; Yu et al, 1997).

The published data concerning the use of cytokines in cancer gene therapy have shown tumour regression can occur. However, the models used are usually far removed from a clinical setting. Experimental models that evaluate the antitumour effects of cytokines are often gauged on the ability of cell lines, engineered to express a cytokine, to form tumours in animals, rather than investigating the effect of cytokine gene transfer to established tumours. If tumours are transduced with cytokine genes, then the level of cytokine production can be expected to be lower than in experimental models, since the efficiency of gene transfer to solid tumours is lower than that achieved in culture. More clinically applicable data might be generated if spontaneously arising tumours, such as those in the transgenic mouse expressing the *neu* oncogene (Sacco et al, 1996), are treated.

Selection of the most suitable cytokine for therapy may be difficult because it appears, at least from the reports in this section, that the effectiveness of cytokines may vary between studies using the same model. In addition, many cytokines may not possess antitumour properties, for example, studies in a B16 tumour model have shown that of 10 cytokines investigated, only IL-2 led to a significant reduction in tumorigenicity (Dranoff et al, 1993).

1.9. Suicide gene therapy

When expressed in cells, suicide genes can lead to direct cytotoxicity (toxin gene therapy) or to conditional cytotoxicity (enzyme / prodrug systems). Enzyme / prodrug systems have been studied more extensively since they can induce bystander killing on neighbouring cells without the suicide gene. Both systems are described below, and the mechanism of bystander killing is considered further in section 1.11.

1.9.1. Toxin gene therapy

Diphtheria toxin (DT; from *Corynebacterium diphtheriae*) is a dimeric toxin consisting of α and β subunits. The α subunit is an ADP-ribosyltransferase which ADP-ribosylates elongation factor 2, preventing protein synthesis. Transgenic mice expressing the α subunit cDNA specifically in pancreatic acinar cells have an abnormal pancreas at birth due to cell ablation (Palmiter et al, 1987).

Ricin and abrin are plant lectin toxins which, like DT, also have α and β subunits. The β subunit is required for binding and entry while the α subunit mediates toxicity by depurination of ribosomal RNA, preventing protein synthesis. Killing of mammalian cells, in culture, following exposure to these proteins has been demonstrated (Hughes et al, 1996).

It is therefore likely that the cDNA encoding the α subunit of these toxins could be used for cancer gene therapy. However, cytotoxicity arises immediately after translation, making the system difficult to control and it is likely that only the cells expressing the toxin would be killed, hence there would be no bystander effect.

1.10. Enzyme / prodrug systems

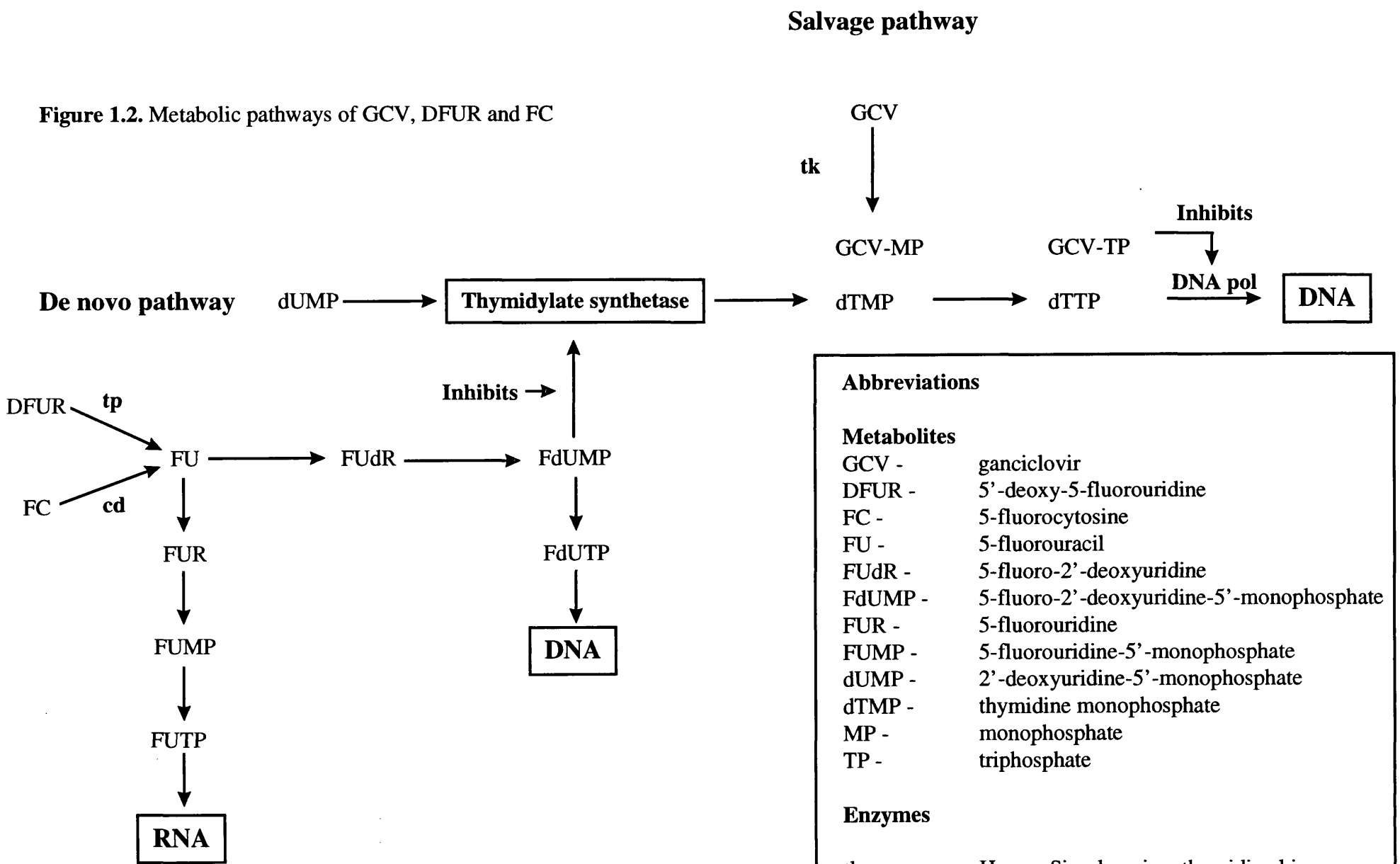
In this form of suicide gene therapy, the cDNA for an enzyme which can convert a prodrug to a toxic metabolite(s) is introduced and expressed in cells. Separately, the enzyme and prodrug are relatively non toxic but in combination the metabolites produced induce cell death. Enzyme / prodrug systems allow conditional toxicity and these two stage strategies are popular in cancer gene therapy. Many combinations exist and details for three are given below.

1.10.1. Herpes Simplex virus thymidine kinase / ganciclovir (tk / GCV) system

Mammalian thymidine kinase (EC:2.7.1.21) is part of a salvage pathway that enables cells to phosphorylate and reclaim thymidine that would otherwise be degraded (Elion, 1980). Herpes Simplex virus thymidine kinase (tk; deoxynucleoside kinase; EC:2.7.1.75) is a comparable enzyme but also phosphorylates purine deoxynucleoside analogues that are poor substrates for the cellular kinases (Elion, 1980). This difference in substrate specificity has been exploited for the treatment of HSV infections (Elion, 1980; Cheng et al, 1983) using ganciclovir (GCV; 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; Cymovene) or acyclovir (ACV; 9-(2-hydroxy ethoxymethyl) guanine), although GCV is more effective (Golumbek et al, 1992).

The tk / GCV system is the most extensively studied of such two stage therapies. tk activates GCV, by phosphorylation of an acyclic, pentose like moiety, yielding GCV monophosphate (Cheng et al, 1983; Field et al, 1983). This reaction is rate limiting in the production of the toxic form of the drug. Further conversion to di- and tri-phosphate forms is catalysed by endogenous cellular kinases (figure 1.2.). GCV triphosphate (GCV-TP) is a toxic metabolite and competes with deoxyguanosine triphosphate (dGTP) for incorporation into

Figure 1.2. Metabolic pathways of GCV, DFUR and FC



nascent chains of DNA in proliferating cells. αDNA polymerase may bind irreversibly to this site, causing chain termination and hence cell death (Cheng et al, 1983).

Clinically GCV is a relatively non toxic prodrug at therapeutic concentrations and this has attracted attention to tk for use in cancer gene therapy. Moolten et al (1986) demonstrated neoplastic murine cell lines transfected with the tk gene were highly sensitive to ganciclovir and subsequently others have made similar observations in a variety of cell lines (Golumbek et al, 1992; Abe et al, 1993; Barba et al, 1993; Kato et al, 1994; Smythe et al, 1994). In each report there was up to 1000-fold difference in sensitivity between tk transfected cells and parental cells. Extension of these studies to animal models has demonstrated that tumours derived from cell lines expressing the tk gene, including melanoma (Vile et al, 1993), glioma (Barba et al, 1993; Kruse et al, 1997), myeloma (Dilber et al, 1996), lung cancer (Osaki et al, 1994), hepatocellular carcinoma (Kuriyama et al, 1997) and large (>4cm) adenocarcinoma (Plautz et al, 1991) can regress with GCV treatment.

Established tumours, derived from cell lines, have been targeted with the tk gene and subsequent treatment with GCV resulted in tumour regression (Culver et al, 1992; Perez-Cruet et al, 1994; Chen et al, 1995b). This was an important observation since only a fraction of the tumour expressed tk and indicated that bystander killing had occurred. A review of the mechanisms of bystander effects is provided in section 1.11. The tk / GCV system is the subject of several clinical trials (Oldfield, 1993; Culver et al, 1994; Raffel, 1994; Freeman et al, 1996) and preliminary data suggest that this enzyme / prodrug combination can, in some cases, lead to therapeutic benefit (Culver et al, 1996).

1.10.2. Thymidine Phosphorylase / 5'-deoxy-5-fluorouridine (tp / DFUR) system

Thymidine phosphorylase (tp; platelet derived endothelial cell growth factor; pyrimidine phosphorylase; thymidine orthophosphate deoxyribosyl-transferase; EC: 2.4.2.4) is a human enzyme involved in the reversible formation of thymidine and orthophosphate from thymine and deoxyribose-1-phosphate (Wadler et al, 1990). Leukocytes and platelets express tp, where the primary function appears to be the regulation of plasma thymidine levels (Chu et al, 1990).

tp also plays a role in angiogenesis (Sumizawa et al, 1993) and since rapidly growing tumours require formation of new capillaries, elevated levels of tp activity are seen in several different cancers, including colon, stomach, bladder and ovarian carcinomas (O'Brien et al, 1996). This association provides an opportunity for targeted chemotherapy using 5'-deoxy-5-fluorouridine (DFUR; Furtulon; Doxifluridine) which is converted, by tp, to 5-fluorouracil (FU).

FU is a base analogue which has been used as a chemotherapeutic agent for many years (Birnie et al, 1963). FU is converted, by cellular enzymes, to toxic metabolites which lead to cell death by interfering with RNA and DNA synthesis (see figure 1.2.). FUTP affects maturation and processing of messenger and ribosomal RNA hence inhibits protein synthesis. FdUTP is a nucleotide analogue which becomes incorporated into DNA and interferes with DNA synthesis. FdUMP (5-fluoro-2'-deoxyuridine-5'-monophosphate) inhibits thymidylate synthetase by covalently binding to N^5N^{10} methylene tetrahydrofolate to form a ternary enzyme-inhibitor-cofactor complex. This irreversible inhibition prevents DNA biosynthesis (review, Springer and Niculescu, 1996). Therefore, FU can lead to apoptotic cell death by mechanisms both dependent and independent of the cell cycle (Schipotin et al, 1994; Crystal et al, 1997).

FU has been successfully used to treat some tumours, however, systemic toxicity is seen in humans and mice at a dose of 15mg / kg, which can limit the therapeutic effect. Toxic symptoms include gastrointestinal tract ulceration and bleeding, and occasionally cardiovascular complications (Connolly et al, 1983; Crystal et al, 1997). In addition, cancer cells often become resistant to FU and this has been attributed to reduced levels of tp enzyme (tp converts FU to FUR and FUdR, see figure 1.2.) in the surviving tumour cell population (Peters et al, 1987). Ideally a less toxic alternative that can be applied at a high dose would be preferred.

DFUR is a relatively non toxic nucleoside analogue which is converted, by endogenous tp, to FU (figure 1.2.). In humans and mice doses of up to 1200mg / body and 200mg / kg respectively, have been administered without toxicity (Yamamoto et al, 1996; Connolly et al, 1983). This should result in an increased level of toxic metabolites within the tumour. DFUR treatment of Ehrlich Ascites tumour in mice (Connolly et al, 1983) and breast tumours in humans (Yamamoto et al, 1996) suggests that DFUR is more effective and less toxic than FU. However, DFUR is not always effective against tumours, and this has been attributed to the variation in levels of endogenous tp activity (Fujimoto et al, 1985).

The cDNA for tp was cloned in 1993 (Sumizawa et al, 1993) which has allowed introduction and expression of the tp gene in cells in culture. Human epidermal carcinoma (KB) cells (Haraguchi et al, 1993), human breast cancer (MCF-7) cells (Patterson et al, 1995) and lung cancer (PC9) cells (Kato et al, 1997) have been transfected with tp cDNA and in each case there was an increased sensitivity to DFUR.

1.10.3. Cytosine deaminase / 5 fluorocytosine (cd / FC) system

Cytosine deaminase (cd; EC: 3.5.4.1) is a prokaryotic enzyme that deaminates cytosine and the base analogue fluorocytosine (FC) to uracil and fluorouracil, respectively (Austin and Huber, 1993). No similar enzymic activity is found in mammalian cells and clinically FC has been used extensively to control bacterial and fungal infections, including *Cryptococcus* and *Candida* (Hirschowitz et al, 1995).

Bacterial conversion of a relatively non toxic prodrug to a toxic metabolite has attracted attention to cd for use in gene therapy. While FU has a narrow therapeutic window and is systemically toxic at doses of >15 mg / kg in humans, no toxicity is seen with FC at doses of 200mg / kg / day for 8 weeks (Huber et al, 1995). Higher doses of FC do cause toxicity, but this is mainly due to uptake of FU produced by bacteria in the intestine (review, Mullen, 1994). This difference in toxicity between prodrug and drug allows high local concentrations of FU to be produced by treating tumours expressing the cd gene with FC, (FU is also produced by the tp / DFUR system and its further metabolism has been described in section 1.10.2 and figure 1.2.).

The cd / FC strategy has been successfully used to kill mammalian cells, for example, Huber et al (1993) demonstrated WiDr (colorectal carcinoma) cells expressing the cd gene required 27μM FC to reduce survival by 50%, approximately 1000-fold less than parental cells. This is well below the peak plasma concentration that can be achieved in humans (approx. 700μM FC; Huber et al, 1995).

Tumours derived from cell lines expressing the cd gene, including mouse mammary adenocarinoma (Consalvo et al, 1995), human colorectal carcinoma (Huber et al, 1993; Huber et al, 1995; Trinh et al, 1995) and colon carcinoma (Hirschowitz et al, 1995), have been shown to regress with FC treatment. In some instances local FU concentrations in the tumour

exceeded 400μM (Huber et al, 1994). Since FC is less toxic than FU, treatment can be given for longer periods of time leading to greater tumour regressions and an increased number of tumour cures (Consalvo et al, 1995). Longer treatment may also enhance the immune response towards tumour cells (section 1.11.5.; Consalvo et al, 1995).

More recently adenoviral delivery (section 1.15.2.) of the cd gene to liver metastases derived from colorectal carcinoma cell lines, in combination with FC treatment, has been shown to result in significant tumour regressions (Ohwada et al, 1996). Observations such as these have led to a clinical trial using the cd / FC enzyme / prodrug system for the treatment of metastatic colon cancer of the liver (Crystal et al, 1997).

1.11. Bystander effects associated with enzyme / prodrug systems

Enzyme / prodrug systems can lead to bystander killing of cells with and without the therapeutic gene, an effect that has also been described previously in association with enhancement of the immune system (section 1.8.). However, the mechanism of bystander effects associated with enzyme / prodrug systems appears to be mediated by toxic metabolites as well as components of the immune system. Since only a low level of transduction can be achieved with current delivery systems (Caruso et al, 1993; O'Malley et al, 1995; Calvez et al, 1996), it will be important to understand the mechanism by which bystander killing occurs. The suggested mechanisms of bystander effects of enzyme / prodrug systems is considered below.

1.11.1. Possible mechanisms for culture and *in vivo* bystander effects

A physical property of all prodrugs used for suicide gene therapy is that they should be able to cross the cell membrane. For example, FC crosses the membrane by diffusion (Haberkorn et al, 1996b), while GCV is carried by a nucleoside transporter (Haberkorn et al, 1996a). For a bystander effect involving transfer of toxic metabolites to occur, metabolites produced by the suicide enzyme must pass to neighbouring cells. If these metabolites can leave the transfected cell and enter the untransfected cells by crossing the plasma membrane, which might be expected in the case of FU (see section 1.10.2.), then no specialised mechanism is required. However, if the metabolites produced by the transfected cells are unable to cross the membrane, then transfer must occur by a different pathway.

It is possible that in the tk / GCV system, phosphorylation of GCV (figure 1.2.) prevents the toxic metabolites from crossing the membrane. The suggested mechanisms for spread of toxic metabolites to tk^- cells include phagocytosis of apoptotic vesicles liberated from dying GCV treated tk^+ cells (Freeman et al, 1993) and transfer of GCV phosphates through gap junctions, by a process known as metabolic co-operation (Bi et al, 1993). This is a controversial issue and evidence for each mechanism is considered below.

1.11.2. Transfer via apoptotic vesicles

Freeman et al (1993) have demonstrated that in mixed cultures of 10% tk^+ and 90% tk^- cells, almost all cells are killed with GCV treatment. In a subsequent experiment, the lipophilic membrane dye, pKH26 (red fluorescence with 488nm excitation), was used to label tk^+ human colorectal carcinoma cells, while tk^- murine tumour cells could be identified by a mouse specific FITC labelled antibody. When mixed cultures of these cells were treated with GCV,

apoptotic vesicles were released from tk^+ cells and their presence was detected in neighbouring tk^- cells. From these experiments it was concluded that toxicity could be transferred via phagocytosis of apoptotic vesicles (Freeman et al, 1993).

Apoptosis and necrosis are two methods through which cell death can occur. Necrosis is a relatively uncontrolled progressive degradation that occurs after death and is normally associated with severe changes in environmental conditions, for example, hypothermia, hypoxia, ischemia or complement attack. Membrane integrity is reduced, causing swelling and rupture of the membranes surrounding the cell and the organelles. Release of lysosomal enzymes results in degradation of cellular components (review, Walker et al, 1988).

Apoptosis, however, is a well controlled process and occurs in two distinct phases. In the latent phase, chromatin compacts to dense masses that associate with the nuclear envelope. The cytoplasm of the cell condenses and DNA undergoes double stranded cleavage at linker regions between nucleosomes to produce fragments which are multiples of 185 bp. Cleavage is specific and associated with a Ca^{++}/ Mg^{++} dependent endonuclease (Kerr et al, 1972; Arends et al, 1990). This phase is variable in length lasting from hours to days (Lazebnik et al, 1993).

The active phase occurs rapidly, in as little as an hour, and is characterised by extrusion of vesicles from the main body of the cell. These apoptotic vesicles vary in size, contain intact organelles and may or may not contain fragments of chromatin. Vesicles are rapidly engulfed by neighbouring cells which prevents the release of macromolecules, such as lysosomal enzymes, that could lead to toxic consequences (Savill et al, 1993).

In support of the role of apoptotic vesicles in transfer of toxicity, the presence of apoptotic bodies in neoplastic cells has been observed (Kerr et al, 1972) and treatment of cells with cytotoxic agents, such as cisplatin or ricin, has been shown to induce shedding of apoptotic vesicles within 24 hr (Cece et al, 1995; Hughes et al, 1996). However, if apoptotic bodies were involved, it is likely that vesicle transfer would occur by phagocytosis, rather than

fusion. This would result in vesicular contents ending up, not in the cytoplasm, but in the lysosomal compartment. Even if toxic metabolites were not degraded, there would still be a membrane(s) to cross to reach the cytoplasm. The role of vesicles in transfer is investigated further in chapter 3.

1.11.3. Transfer via gap junctions

Mixed cultures of human fibrosarcoma cells with and without the tk gene only show a bystander effect when cells are in contact (Bi et al 1993). In sparse cultures only tk^+ cells were killed and subsequent experiments showed that incorporation of ^3H GCV to tk^- cells only occurred when they were in contact with tk^+ cells. These results suggested that the mechanism of transfer of toxicity involved metabolic co-operation requiring the presence of functional gap junctions (Bi et al 1993).

Metabolic co-operation between coupled cells has been readily demonstrated in other culture systems. Fibroblasts taken from patients with Lesch-Nyhan disease are deficient in hypoxanthine phosphoribosyl transferase ($HPRT^-$). This enzyme is involved in the salvage pathway of purine nucleotide synthesis from hypoxanthine. If the normal pathway of nucleotide biosynthesis is inhibited by aminopterin, then these mutants are unable to grow in full medium supplemented with hypoxanthine, but can be rescued when cultured in contact with $HPRT^+$ cells. Mutant cells undergo prompt phenotypic reversion when separated, even though the enzyme has a long half life and this suggests that transfer of nucleotides, rather than enzyme, had occurred (Cox et al, 1970).

Moreover, $HPRT^-$ cells are resistant to the purine analogue thioguanine (TG) when cultured alone but in mixed cultures with wild type cells, become sensitive to the toxic effects of TG (Fujimoto et al, 1971). This effect has been termed the kiss of death and is directly

analogous to the bystander effect seen with the tk / GCV system, supporting metabolic co-operation as a mechanism of transfer of toxicity.

If gap junctions are involved in the tk / GCV bystander effect then the use of this enzyme / prodrug system may be limited to tumours that have high levels of gap junctional communication. With this in mind, a brief review of the structure, function and role of gap junctions is provided in section 1.12.

1.11.4. Possible mechanisms for *in vivo* bystander effects

Cell killing *in vivo* is more effective than killing in culture, in some models. For example, Barba et al (1993) demonstrated that all C6 tk^+ glioma cells could not be killed by continuous GCV in culture, but brain tumours formed from these cells could be cured. In this study tk^+ cells were seeded (in culture) at relatively low density and it is possible that some cells acquired resistance to GCV (by disruption or methylation of the tk gene or promoter sequences), but due to the lack of cell-cell contact, were not killed by neighbouring tk^+ cells that retained the functional gene. If resistant cells did arise *in vivo* then additional factors must have led to bystander killing. Experiments have shown a functional immune system and damage to tumour vasculature are important for effective tumour clearance.

1.11.5. Role of the immune system

There is an increasing amount of evidence to suggest that a functional immune system is required for tumour eradication. For example, lung metastases, derived from a B16 melanoma cell line, have been shown to regress in syngeneic but not nude mice (Vile et al, 1994b). In a separate study, rats with established 9L tk^+ brain tumours were cured with GCV

and, while all naive animals succumbed to a subsequent rechallenge of parental cells in the contra-lateral flank of the body and the brain, pre-treated (9L tk^+ / GCV) animals did not develop tumours, in some cases. Immunity was also shown to be specific since protection against 9L cells and not other tumorigenic cell lines was observed (Barba et al, 1994) suggesting that the response was T-cell mediated. The role of the immune system has been implied by others who have shown extensive infiltration of the residual tumour by $CD8^+$ lymphocytes, to a lesser extent $CD4^+$ and $CD3^+$ cells, granulocytes and macrophages (Caruso et al, 1993; Consalvo et al, 1995; Gagandeep et al, 1996; Yamamoto et al, 1997).

The particular role of some of these immune cells has been demonstrated by the use of antibody blocking experiments. In colon carcinoma and adenocarcinoma models granulocytes are required for initial tumour inhibition, $CD4^+$ helper cells are required for rejection of tumour cell rechallenge and $CD8^+$ cytotoxic cells function in both roles (Chen et al, 1995b; Consalvo et al, 1995). This suggests that $CD4^+$ and $CD8^+$ cells are required for efficient generation of immune memory.

Release of cytokines from GCV treated tumours, derived from cell lines engineered to express the tk gene has been documented, but appears to depend on the particular tumour cell line. For example, tk^+ tumours derived from fibrous histocytoma cell lines release interleukin (IL) 1α / β, IL-6, IL-8 and the chemokine RANTES after GCV treatment, but tk^+ ovarian carcinomas released IL-1α / β and IL-8 (Abboud et al, 1996). TNFα has been associated with GCV treatment of experimental tk^+ human colon carcinoma (Freeman et al, 1995), while IFNγ and GMCSF are released from tk^+ murine fibrosarcoma (Ramesh et al, 1996). In separate studies, these cytokines have been shown to exhibit antitumour activities in experimental models (see section 1.8.2.).

Due to the apparent importance of the immune system, some reports have documented combination of suicide and cytokine genes, for example tk and IL-2 (Chen et al, 1995b) or cd

and IL-6 (Mullen et al, 1996), for treatment of experimental tumours. This approach has led to an increased number of tumour cures compared to either system alone and it has been suggested that cell death caused by the enzyme / prodrug combination may increase antigen presentation, while the cytokine activates local T-cells (Mullen et al, 1996). Consistent with this hypothesis, GCV mediated killing of experimental renal carcinomas, expressing the tk gene, leads to increased expression of MHC-I antigen (Barba et al, 1994).

1.11.6. Damage to tumour vasculature

Fast growing tumours require a good blood supply and damage to vasculature may lead to tumour ischemia. It has been demonstrated that retroviral vectors transduce endothelial cells of tumour capillaries in the brains of rats (Ram et al, 1993) and endothelial cells may be coupled, by gap junctions, to surrounding cells (Kam et al, 1986). After tk transduction and GCV treatment, tumour haemorrhaging has been observed (Perez-Cruet et al, 1994) and Doppler imaging has shown a five fold decrease in the number of tumour blood vessels in a subcutaneous glioma model (Ram et al, 1994).

1.12. Gap junctional intercellular communication (GJIC)

It has been suggested that metabolic co-operation is required in the tk / GCV bystander effect (see section 1.11.3.). Therefore a basic understanding of the structure and function of gap junctions may be necessary to complete an investigation of transfer of toxic metabolites from tk^+ to tk^- cells (Chapter 3).

GJIC is necessary in complex, differentiated organisms to co-ordinate the activities, differentiation and growth of component cells. Gap junctions allow ions and small molecules

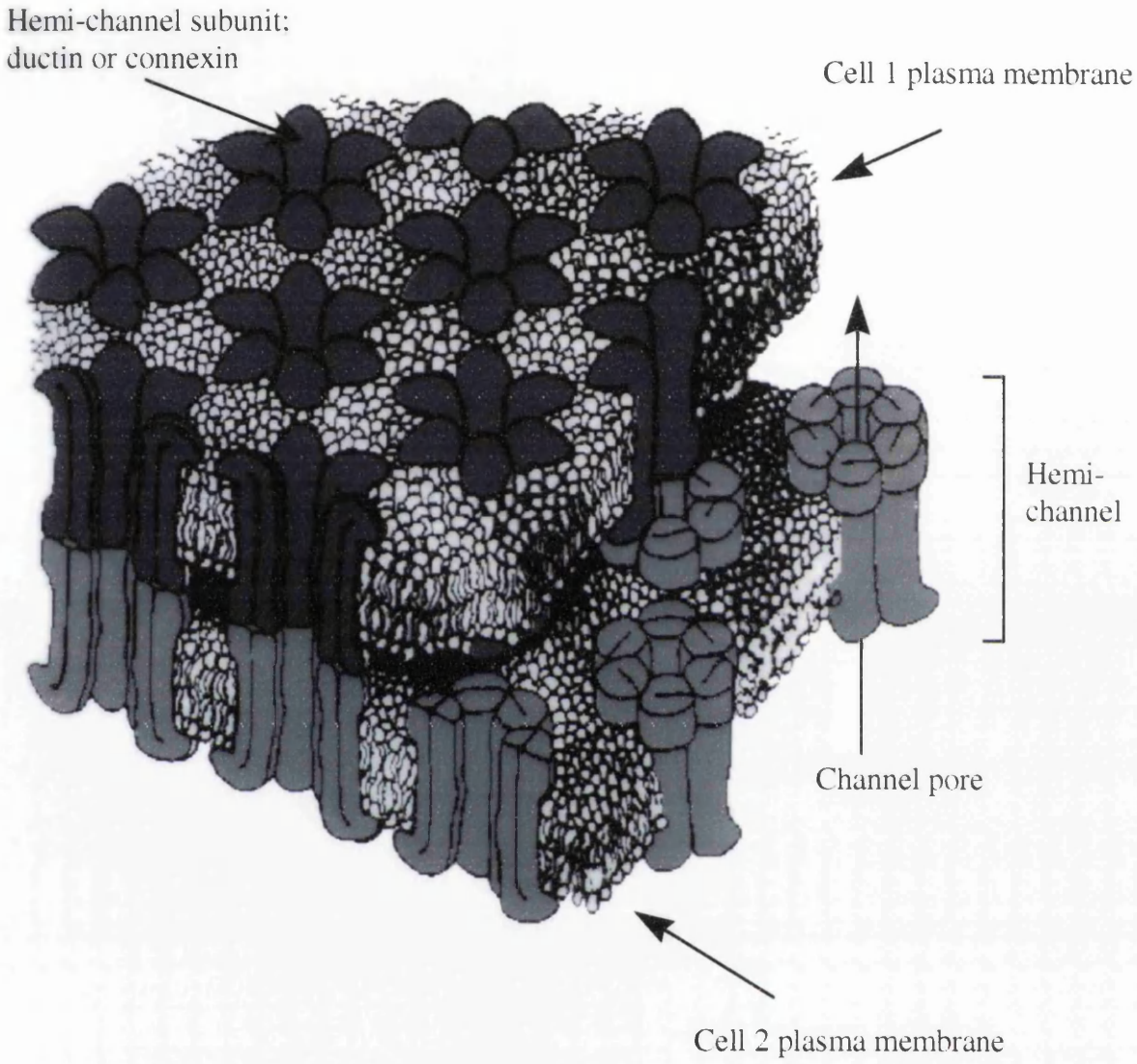
(Mr of <1000) to pass between coupled cells by diffusion, but macromolecules such as proteins and nucleic acid cannot pass to neighbouring cells. Exchange of low molecular weight solutes is, however, restricted to distinct communication compartments, where cells within a compartment are coupled to each other, but not to cells within neighbouring compartments (review, Pitts and Finbow, 1986). The role of junctional communication within excitable tissues and during tumorigenesis is considered in section 1.12.2., but to understand gap junctions better, some basic structural information is provided below.

1.12.1. Gap junction structure

The gap junctional channel is composed of two hemi-channels termed connexons. Each hemi-channel joins end to end with another, provided by the apposing membrane of an adjacent cell. Each connexon is composed of six subunits, arranged symmetrically around an axial water filled channel with a diameter of 1-2 nm (figure 1.3.). However, there has been a controversy as to what protein is the principal channel component of the connexon (review, Finbow and Pitts, 1993). The debate revolves around two proteins, ductin and connexin.

Ductin is a 16 kDa protein which is found in cells of diverse origin, for example, in plants, fungi, vertebrates and invertebrates (Finbow et al, 1984; Finbow et al, 1993). Isolated gap junctions or gap junction regions in tissue sections are bound by antibodies to invertebrate ductin (Buultjens et al, 1988; Leitch and Finbow, 1990). GJIC is inhibited when anti-ductin antibodies are injected into mammalian or invertebrate cells (Finbow et al, 1993). However, ductin also forms a major component of the vacuolar ATPase proton channel (Mandel et al, 1988) and it is not possible to produce ductin knockouts that are not lethal. Evidence therefore remains circumstantial.

Figure 1.3. Schematic representation of the proposed gap junction connexon



Based on diagram by Makowaki et al. 1977.

Several studies have demonstrated that junctional communication of poorly coupled cells can be increased by introducing connexin genes (Mesnil et al, 1996; Elshami et al, 1996; Shinora et al, 1996) and reports such as these lend support to the requirement for connexins in gap junction formation. However, introduction of connexin genes into MCF-7 mammary carcinoma cells that lack a functional endogenous gene and are not coupled, fails to introduce communication (Pitts, unpublished data) and no connexins have been identified in invertebrates, despite successful isolation of gap junctions from such organisms (Finbow et al, 1984).

It is therefore possible that ductin forms the major protein of the gap junction, while connexins play a largely regulatory role.

1.12.2. Communication compartments

Organisation of tissues into communication compartments allows cells within a coupled population to share the same low molecular weight signals, while cells in different compartments can share different signals. Sharing such signals may cause cells to act in synergy or may place homeostatic pressure on aberrant cells.

Gap junctions, acting as electrical synapses, allow action potentials to spread quickly and uniformly within excitable tissues such as the heart and smooth muscle. In other excitable tissues however, such as skeletal muscle and the CNS, gap junctions are lost during the terminal stages of differentiation (review, Pitts and Finbow, 1986) as electrical coupling would produce unacceptable all or nothing responses.

The homeostatic pressure which arises due to communication within a coupled cell population can suppress the expression of individual cell phenotypes. For example, in culture, normal cells can suppress the aberrant growth of certain transformed cells, providing gap

junctions are formed between the two cell types (Mehta et al, 1986). The mechanism of this suppression may involve the uniform distribution (hence dilution) of second messengers associated with the transformed phenotype between cells of the coupled population. It has therefore been suggested that for a tumour to form, cells must lose junctional communication (review, Yamasaki, 1990). However, it has become apparent that it is rare for tumour cells to actually lose the capacity to communicate, but in most cases communication is markedly reduced. For example, reduction of junctional communication has been observed during the progression of mouse squamous cell carcinomas to spindle carcinomas (Holden et al, 1997). In this model, B9 squamous cells represent an intermediate stage of progression and have high levels of GJIC, but further progression to CarB spindle cells results in the loss of the adhesion protein, E-cadherin, and a >5-fold reduction in junction communication. Other studies suggest that an important factor in tumour formation appears to involve reduction or loss of communication between the tumour cells and the surrounding normal cells, rather than between tumour cells (review, Yamasaki, 1990).

Therefore, before considering the application of the tk / GCV enzyme / prodrug system, it will be important to establish the mechanism of transfer of toxic metabolites from tk^+ to tk^- cells. If high levels of GJIC are required for the spread of toxic metabolites then it is possible that the tk / GCV system will not be suitable for treatment of many tumour types.

1.13. Vectors

In culture, vectors such as retroviruses can achieve a high efficiency of gene transfer to cells because most cells are readily accessible and are cycling, a requirement for retroviral infection. This contrasts to the dense 3 dimensional structure of solid tumours, where access to the majority of the cells is restricted, cell division is slow and the level of gene transfer is low.

Ideally cells would be explanted from tumours and therapeutic genes introduced to the cells while in culture. This *ex vivo* approach has been adopted for transfer of IL-2 and TNF-α cytokine genes to TILs (Rosenberg et al, 1993), with treatment being beneficial in some cases.

Explanting cells to culture is not always successful, since primary cultures cannot always be established and cell numbers cannot always be increased sufficiently to be of clinical value. Another consideration is that the antigenic profile of the cells may change during the period of culture and when reintroduced to the patient, the expanded cell population may be rejected. The *ex vivo* approach is also labour intensive and time consuming, since expansion of, for example, TILs, requires up to 10 weeks (Rosenberg et al, 1990). For cancer gene therapy to be successful, it is likely that gene transfer will have to be accomplished *in vivo.* Some of the current delivery methods being investigated are considered below, and the problems of gene delivery are discussed further in section 1.17.

1.14. Non viral delivery

1.14.1. Naked DNA

Introduction of naked DNA by direct intratumoural injection has been suggested as a method of gene transfer (Vile et al, 1993) because DNA is of low immunogenicity and, unlike some viral vectors (section 1.15.), allows tissue / tumour specificity of promoters (section 1.16.) to be retained. However, transfer efficiency is low and only in some instances has a therapeutic effect been observed (Vile et al, 1993; Vile et al, 1994b).

To try to improve the efficiency of gene transfer, Nishi et al (1996) used 'electrogene' therapy in which intra-arterial plasmid DNA coding for β-galactosidase was injected and short pulses of electricity were used to increase efficiency of uptake. However, this approach

succeeded in only doubling the level of β-gal activity in the tumours compared to when control plasmid was introduced by this approach.

1.14.2. Liposomal delivery

Cationic liposomes are positively charged lipid based complexes that can condense DNA spontaneously to form complexes with high affinity to cell membranes. Endocytosis of complexes, followed by disruption of the endosomal membrane appears to be the major mechanism of gene delivery (Gao and Huang, 1995). Liposomes are non immunogenic, safe and may preferentially target tumours since it has been suggested that they are able to leak from newly forming capillaries which are found in developing tumours (review, Boulikas, 1996).

Liposomes have been used to deliver therapeutic genes, such as the tk gene, to cultured cells and experimental tumours, including pancreatic cancer (Aoki et al, 1997) and B16 melanoma (Calvez et al, 1996). Although gene transfer to 10% of B16 tumour cells was achieved in culture, therapeutic doses of prodrug (≈20μM GCV) killed only 20% of cells (Calvez et al, 1996). A bystander effect using the tk / GCV system has previously been demonstrated in these cells in culture, therefore it is possible that the level of tk expression was low or not sustained. The use of viral delivery may achieve higher levels of expression and this method is considered below.

1.15. Viral delivery

1.15.1. Retroviruses

Retroviruses represent a very efficient method of gene transfer to cells in culture, with transduction rates of 90% in some fibroblastic cell lines, but since tumours have long replication cycles and these vectors persist for a short time, transduction efficiency *in vivo* may be <0.1% in some cases (Short et al, 1990). However vector producer cells (vpcs) have been shown to be able to achieve gene transfer to 10% of cells in experimental tumours (Short et al, 1990). Vpcs can allow the production of recombinant retroviral particles for up to 14 days *in vivo*, but after this time they are removed by an immune response (Moorman et al, 1994).

Vpcs are constructed from a cell line, often fibroblastic, which is engineered to express the viral genes, gag, env and pol, in trans while a helper virus expresses the viral genes required to generate an empty retroviral particle. This cell line is also stably transfected with a plasmid carrying the remaining retroviral genes, in the 5' and 3' LTRs. Expression of the foreign nucleic acid is often driven by the promoter regions in one of the viral LTRs. Therefore the vpcs derived, produce replication defective viral particles that can transduce dividing cells and express the therapeutic gene. Injection of vpcs carrying the tk gene to experimental tumours in animals, combined with GCV treatment, has led to a significant tumour regressions, without apparent toxicity to surrounding normal tissue (Culver et al, 1992; Caruso et al, 1993; Ram et al, 1993a; Izquierdo et al, 1995; Ram et al, 1993b). Several clinical trials are on-going using this method of gene transfer (Oldfield et al, 1993; Culver et al, 1994; Izquierdo et al, 1996).

There are, however, several problems with retroviruses, for example, they only infect dividing cells. Although this has made them a popular choice for treatment of brain tumours

where the surrounding tissue is mainly quiescent (Izquierdo et al, 1995), tumour cells are often not dividing and surviving untransduced cells may repopulate the tumour (Maron et al, 1996; Cool et al, 1996). Furthermore, in some cell lines expression of the tk gene has been demonstrated after retroviral transduction in culture, but not when delivery was attempted in tumours derived from these cell lines (Zhang and Russell, 1997). It is possible that the tk gene in the vpcs used was unstable, or the cells within the tumour were not cycling at a sufficient level to permit infection by these retroviruses.

To achieve expression of the therapeutic gene, viral sequences must integrate into the host genome. This event is non specific and although the risk of oncogene activation, through insertional mutagenesis is remote (Moolten et al, 1992), integration into silenced regions of the genome may reduce therapeutic gene expression. Blaese et al (1993) reported 100-fold variation in levels of expression of therapeutic genes and attributed the differences to position of gene insertion.

A concern for the future use of retroviruses are the observations that viral titre has been reduced when tissue specific promoter elements are introduced to the vector, and that tissue specific expression (section 1.16.) is not always retained (Kuriyama et al, 1991; Vile et al, 1994a).

1.15.2. Adenoviruses

Adenoviruses have been investigated as a method of gene delivery because, unlike retroviruses, they infect a wide range of cells, independent of cell cycle (review, Vile et al, 1994c). Adenovirus can be prepared to a high titre and can superinfect a cell which may lead to high levels of gene expression (Kaneko et al, 1995). Currently most adenoviruses used in gene therapy have the E1 and E3 regions of the genome deleted and replaced with foreign

genetic sequences. E1, but not E3, is essential and must be supplied in trans by a producer cell line. The particles released from this cell line are replication defective.

Intratumoural injection of recombinant adenovirus has been used to deliver the tk gene to experimental tumours, and combined with GCV treatment, can lead to long term animal survival (Perez-Cruet et al, 1994; Chen et al, 1994; Colak et al, 1995). More recently clinical trials have been initiated (Crystal et al, 1997; Alvarez et al, 1997).

Despite these promising reports there are still several issues that require attention. Adenoviruses can infect many cell types and as such can cause non specific cytotoxicity which appears to vary depending on the cell line or the animal model. For example, toxicity in cultured astrocytes has been shown to occur at a multiplicity of infection (MOI) of 10 (Maron et al, 1995), while survival of C6 glioma cells was unaffected at an MOI of 125 (Chen et al, 1994). In baboons intracranial injection of $>10^7$ pfu resulted in severe toxicity (Goodman et al, 1996) whereas in rhesus monkeys injected with up to 10^{11} pfu, to the same site, showed no significant toxicity (Smith et al, 1997b). The variation in cytotoxicity observed may be due to differences in binding and entry of adenovirus that have been suggested from culture studies (Bonnekoh et al, 1996; Smythe et al, 1995; Chen et al, 1996b).

Adenoviruses elicit an immune response which may inhibit further vector administration. However, recent findings show antibodies to one serotype do not always cross react with another and it may be possible to circumvent the immune response by sequential delivery of a different recombinant adenovirus serotypes (Kass-Eisler et al, 1996). Alternatively deletion of further adenoviral genes may reduce the immune reactivity of the virus (Chen et al, 1996b) and this approach may also prevent disruption of tissue specific therapeutic gene expression that has been reported (Ring et al, 1996).

1.15.3. Adeno-associated virus (AAV)

AAVs are single stranded parvoviruses that can be prepared to high titre. Infection is not dependent on cell division and due to their small size (≈20nm compared to adenovirus of ≈70nm), they may penetrate into tumours more readily. AAVs are non pathogenic in humans and have been used to achieve tissue specific expression of the tk gene in hepatoma cell lines in culture (Su et al, 1997).

However, as AAVs have a small genome, only 4kb can be replaced by foreign genetic sequences and, while wild type viruses have been shown to integrate site specifically to chromosome 19, recombinant AAVs appear to lack this specificity (Ponnzhagan et al, 1997). Therefore there is a theoretical risk of insertional mutagenesis, similar to that when using retroviral gene transfer. Also similar to retroviruses, recombinant AAVs may integrate into silenced regions of the host genome, and expression of the therapeutic gene may be reduced.

1.16. Tumour specific gene expression

In order to achieve expression of a therapeutic gene exclusively in tumour cells, gene transfer must be accomplished to a selected population of cells in culture, or as a targeted approach *in vivo*. *Ex vivo* transduction of cells isolated from a tumour has been described previously in section 1.4.1. In this approach, primary cultures often fail and antigenic changes can occur during adaptation of cells to culture conditions (Rosenberg, 1992). Therefore, it is likely that gene transfer protocols will need to be accomplished *in vivo*.

The requirement for restricted gene expression, if gene transfer is accomplished *in vivo*, has been demonstrated using adenoviral delivery of the tk gene intrahepatically to mice, or intracranially to baboons, where combined treatment with GCV led to death of animals

(Brand et al, 1997; Goodman et al, 1996). In these studies, injection of GCV or adenovirus alone did not lead to animal fatalities. Therefore it is possible that the tk / GCV combination killed dividing endothelial cells causing haemorrhage and death. It has also been suggested that the fatalities were due to killing of non dividing cells (Brand et al, 1997) but further studies are required to investigate this suggestion.

The method of tumour targeting that has been used most frequently is regional delivery of murine retroviruses, because these vectors preferentially infect dividing cells. Studies involving implantation of vector producer cells into experimental liver and brain tumours have demonstrated that transduction was restricted to tumour cells and some endothelial cells (of tumour capillaries) since surrounding normal cells were mostly quiescent (Curaso et al, 1993; Barba et al, 1994). The disadvantage of this method of tumour targeting is that in many human tumours only a relatively small proportion of the cells are dividing at any one time and studies in patients with glioblastoma multiforme have demonstrated that the number of tumour cells transduced was <0.2% (Ram et al, 1995).

To accomplish gene expression in tumour cells following systemic delivery of a vector, further means of targeting are required. However, attempts to engineer retroviral envelope proteins so that they recognise specific cell surface receptors have been hampered by incorrect ligand folding in the viral membrane, resulting in poor entry to tumour cells (review, Chong and Vile, 1996). At the present time the most realistic approach appears to be the use of transcriptional control.

Ideally transcriptional control of therapeutic genes would be accomplished using a promoter that is only active in tumour cells and produces a high level of gene expression. However, most promoter activity is only increased, relative to normal tissue, during carcinogenesis or restricted to specific tissues. For example, carcinoembryonic antigen (CEA) is highly expressed in several cancers of epithelial origin, such as those in the lung, breast and

pancreas, but this protein is also found in normal colonic mucosa (Osaki et al, 1994; Lan et al, 1996). The CEA promoter has been shown to drive expression of the tk gene in CEA positive, but not CEA negative cell lines (Dimaio et al, 1994). Tissue specific expression can be achieved from the tyrosinase (tyr) promoter and a tyr-lacZ construct was shown to express β-gal in 12 of 14 melanomas but not in 12 cell lines from different origins (Vile et al, 1993) The tyr promoter has also been used to direct expression of suicide genes (Vile et al, 1993; Vile et al, 1994a; Hughes et al, 1995).

Several other promoter elements with a level of tissue or tumour specificity have been used for therapeutic gene expression in cell lines. These include the promoters of α-foetoprotein (AFP) for liver cancer (Ido et al, 1995), myelin basic protein (MBP) for gliomas (Miyao et al, 1993), ErbB2 for breast cancer (Harris et al, 1995) and osteocalcin for fibroblastic osteosarcoma (Ko et al, 1996).

There are some reports that suggest the tissue specificity of promoter elements may be reduced, in some cases, when constructs are delivered using retro- and adeno-viral vectors (Vile et al, 1994a; Ring et al, 1996). Furthermore, some promoters, such as AFP, may achieve less than 1% of the expression seen with CMV promoter, which is often used in experimental systems (Kaneko et al, 1995; Kanai et al, 1997).

Another consideration is that targeting expression of suicide genes to tumour cells may reduce the effectiveness of the therapy. For example, damage to tumour capillaries may result in tumour ischemia (Ram et al, 1993a; Ram et al, 1994) but tissue specific targeting may reduce suicide gene expression in the endothelial cells of the capillaries, thereby reducing this part of the bystander effect. Targeting using specific promoters may also reduce the level of gene expression due to the heterogeneity seen in human tumours since some cells may not possess the appropriate transcription factors.

1.17. Problems facing gene therapy

The reports described in this chapter demonstrate that introduction of therapeutic genes to cells in culture, or tumours derived from these cells, can lead to tumour cell death after treatment with prodrug. In addition, most of the approaches described here are currently being investigated as phase I clinical trials. However preliminary clinical data suggest that killing of human tumours is not nearly as successful as that achieved in experimental animals. A summary, which includes some possible problems facing gene therapy, is provided below.

Perhaps the foremost problem is one of gene transfer to tumour cells. It has been demonstrated that the level of retroviral transduction achieved in tumours derived from a C6 glioma cell line is <0.1%, but this can be improved 100-fold by the use of vector producer cells (vpcs). However these studies were performed under favourable conditions, such as targeting small tumours that have been freshly produced and hence are rapidly growing and susceptible to retroviral infection. Furthermore, the number of vpcs in these models are often greater than the number of tumours cells (Short et al, 1990). It is perhaps not surprising that <0.2% of cells expressed the tk gene following implantation of vpcs into large, slowly dividing tumours such as human glioblastomas (Ram et al, 1995) and that subsequent treatment had little effect in most cases. Cure of small experimental tumours has been observed only when >4% of tumour cell expressed the cd gene (Huber et al, 1994).

It is possible that gene transfer will be more successful when vectors that do not require cells to be in cycle, such as adenovirus, are used. However, to achieve a high level of gene transfer, even in culture, may require a high multiplicity of infection (MOI) which could prove to be toxic. For example, at an MOI of 1, adenoviral mediated delivery of the tk gene was detected in 2% of 9L cells in culture, but cytotoxicity was seen at an MOI of 50. In

humans it is unlikely that even an MOI of 1 can be achieved, since tumours are large and often contain $>10^{11}$ cells, but the maximum dose in current clinical trials is $<10^{10}$ adenoviral particles (Crystal et al, 1997; Alcarez et al, 1997). It is therefore apparent that delivery systems need to be improved, both in terms of gene transfer efficiency and reduced toxicity.

One potential method for reducing toxicity is to target expression specifically to tumour cells, and the most likely way this can be accomplished at present is by the use of transcriptional control (section 1.16.). However this may mean using a weaker promoter and achieving expression in fewer tumour cells, which might be expected to reduce the therapeutic effect.

Due to the problems of therapeutic gene transfer and expression, it may be important to select systems where the therapeutic effect can be amplified. This may be the case in enzyme / prodrug systems but not in antisense strategies. For example, it might be expected that antisense treatment will lack a bystander effect and that gene transfer to every cell within the tumour will be required, which is unlikely to be possible. In addition, the number of effector molecules is low since the only amplification step, in this strategy, is from DNA to RNA. This contrasts to enzyme / prodrug systems that often show a bystander effect and the level of toxicity is relatively high since the processes of transcription, translation and enzymic conversion of prodrug to toxic metabolites amplifies the number of effector molecules.

The immune system has also been shown to be important for tumour clearance and is likely to be an essential feature of any cancer gene therapy protocol. However, further work is required to determine which cytokine(s) is most effective for treatment of tumours since the antitumour effect appears to vary between different experimental models. For example, tumorigenicity of mammary adenocarcinoma cells engineered to express IFNγ was reduced, while IL-6 had no significant antitumour effect (Allione et al, 1994), but in B16 melanoma, the reverse was true (Dranoff et al, 1993).

The current problems that face gene therapy reflect the infancy of this area of research. Each aspect of cell targeting and killing needs to be improved, in particular, gene transfer and bystander effects. If this can be achieved then it is possible that gene therapy may provide a supplementary treatment for cancer. The aim of this project was to investigate the mechanism of bystander effects associated with enzyme / prodrug systems, and these aims are summarised below.

1.18. Aims

The first clinical trials using the tk / GCV system were initiated in 1993. The mechanism of the bystander effect associated with this strategy was not fully understood, although various suggestions had been made. These included spread of toxic metabolites to neighbouring cells without the tk gene, a role of the immune system and damage to tumour capillaries leading to tumour ischemia. The mechanism of transfer of toxic metabolites from tk^+ to tk^- cells was a controversial issue and it was suggested that it may depend on gap junctions (Bi et al, 1993) or phagocytosis of apoptotic vesicles released from dying tk^+ cells (Freeman et al, 1993). During the course of this thesis, it was also suggested that transfer may occur via the medium (Kuriyama et al, 1995).

Spread of toxic metabolites may be an essential feature of enzyme / prodrug systems. Therefore the aim of this project was to determine the mechanism of transfer of toxic metabolites in tk / GCV system. The project was also aimed at investigating the mechanism and efficacy of the tp / DFUR and cd / FC enzyme / prodrug systems, in comparison to the tk / GCV strategy. In addition, as it seems likely that bystander effects will need to be enhanced for successful application of these systems, methods by which they can be optimised were investigated.

Chapter 2. Materials and Methods.

2.1.1. Chemicals

Chemicals used throughout this thesis were of "AnalR" grade and obtained from BDH Chemicals Ltd., Poole, Dorset, England or Sigma Chemical Co. Ltd., Poole, Dorset, England, except those obtained from the suppliers listed below.

Supplier	Chemical
Amersham International Plc., Aylesbury, Bucks, UK.	$\alpha[^{32}P]$-dCTP (3000Ci/mmol) Hybond-ECL Hybond N^+
BRL (UK), Gibco Ltd., Paisley, Scotland.	All restriction enzymes and buffers T4 DNA ligase and buffer
Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.	DNA molecular weight marker (VI)
J. Burrough (FAD) LTD., Witham, Essex, England.	Ethanol
Central Services, Beatson Institute.	Sterile distilled water, PBS, L-Broth, PE buffer (PBS.EDTA buffer)
Difco Labs., Detroit, Michigan, USA.	Bacto-agar and Bacto-tryptone
Gateway Plc., Glasgow, Scotland.	Marvel dried non-fat milk powder
Moravek Biochemicals, USA	6-[^{3}H] cytosine. 0.14mCi/mmol
NEN DuPont, Stevenage, Hertfordshire, England.	5-[^{125}I] Iodo-2'-deoxycytidine. 2200 Ci/mmol.
Oxoid Ltd., Basingstoke, England.	PBS tablets
Pharmacia Biotech Ltd., Milton Keynes, England.	Micro spin columns s-400 HR
Syntex Pharmaceuticals, Maidenhead,	Ganciclovir, Sodium Salt

Berkshire, England.	

2.1.2. Kits

Supplier	Kit
Amersham International Plc., Aylesbury, Bucks, England.	ECL Western Blotting analysis system
Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.	Cell death detection kit
Invitrogen, De Schelp 12, Netherlands.	TA cloning kit
Nucleon, Coatbridge, Scotland, UK.	Mini plasmid preparation kit
Pharmacia Ltd., Milton Keynes, England.	Oligo-labelling kit
Promega, Southampton, England.	Non Radioactive Cell Proliferation kit
Qiagen, Crawley, West Sussex, England.	Maxi plasmid preparation kit Qia-quick Gel Purification kit PCR Purification kit
Stratagene, Cambridge, England.	pCR-Script kit

2.1.3. Water

Distilled water for solutions was obtained from a Millipore MilliRO 15 system, and for nucleic acid procedures it was further purified on a Millipore MilliQ system.

2.1.4. Equipment and Plasticware

Main pieces of equipment are referred to in the appropriate sections. The suppliers of the most commonly used items are listed below.

Supplier	Equipment
Becton Dickinson Labware, Plymouth, Devon, England.	Tissue culture plates (60 and 90mm)
Bibby Sterilin Ltd., Stoney, Staffs.,	Bacteriological dishes (90mm)

England.	
Chance Propper Ltd., Warley, England.	22 x 50mm coverslips
Clarke Electromedical Instruments, Reading, England.	Kwik-fill thin wall glass capillaries
Costar, Cambridge, Massachusetts, USA.	6 and 96 well plates. Disposable cell scrapers Multichannel pipette
Eastman Kodak Co., Rochester, New York, USA.	X-ray film (XAR-5)
Gibco Europe, Life technologies, Paisley, Scotland.	Nunc 1 ml cryotubes. Nunc 0.02μm tissue culture filter inserts 2 well glass chamber slides
Griener Labortechnik Ltd., Dursley, England.	Eppendorf tubes Pipette tips (200 and 1000 μl)
Varian Ltd., California, USA	Bond elute SCX 1ml columns
Whatman International Ltd., Maidstone, England.	DE81 ion exchange paper

2.1.5. Plasmids and bacterial host.

Competent E.coli DH5α cells were obtained from BRL Gibco Ltd., Paisley, Scotland. Below are details of the plasmids used in this thesis and where they were obtained from.

Plasmid	**Resistance marker**	**Origin**
pCDNA 3.1^+	Neomycin	Purchased from Invitrogen, De Schelp 12, Netherlands.
pCDNA 3.1^-	Neomycin	Purchased from Invitrogen.
pCEP4	Hygromycin	Purchased from Invitrogen.
pCDNA 1-TP (Thymidine Phosphorylase)	Neomycin	A gift from Dr. Adam Patterson, MRC radiation unit, Oxford, England.

pL8 -TP (Thymidine Phosphorylase)	-	A gift from Dr. Carl Heldin, Sweden.
pGK-TK (Thymidine Kinase)	Neomycin	A gift from Prof. Alan Balmain, Beatson Institute.
pCD2 (Cytosine Deaminase)	Neomycin	A gift from Dr. Craig Mullen, Houston, Texas, USA.
pSVL-β gal (β-galactosidase marker)	-	A gift from Iain Foulkes, Beatson Institute (cotransfected with pBAG - Neo resistance)
pRc/CMV Cx43 Antisense	Neomycin	A gift from Dr. Alan Lau, Hawaii, USA.

2.1.6. Antiserum

For detection of Connexin 43 in Western Blot analysis the following antibodies were used.

Primary antibody: mouse monoclonal anti-connexin 43 (Zymega Research, San Francisco, CA 94080, USA.)

Secondary antibody: Sheep anti-mouse hp-linked whole antibody (Amersham International Plc., Bucks., England.)

2.1.7. Cell Culture Materials.

Supplier	**Material**
Gibco Europe, Life technologies, Paisley, Scotland.	2.5% (w/v) trypsin Gentamycin Amphotericin B 200mM Glutamine 7.5% Sodium Bicarbonate 100mM Sodium Pyruvate Hepes Buffer 10 x DMEM 1 x SLM
Northumbria Biologicals.	Foetal Calf Serum

Sigma Chemical Co., St Louis, USA.	4% (w/v) Trypan Blue

2.1.8. Cell Lines.

Cell Line	Origin and Description
BHK 21	Syrian Baby Hamster Kidney fibroblast line (Macpherson and Stroker, 1962).
CarB	Mouse spindle cell carcinoma. Established by Prof. Alan Balmain at the Beatson Institute.
CarB-E	Mouse spindle cell carcinoma stably transfected with E-Cadherin by Brenda McGuire at the Beatson Institute.
B9	Mouse epithelial line. Established by Prof. Alan Balmain at the Beatson Institute.
L 929	Mouse fibroblast line (Sanford et al, 1948).
MCF-7	Human mammary epithelial tumour line (Rose and McGrath, 1975).
B95	p53 negative fibroblast line established by Prof. Alan Balmain at the Beatson Institute (From p53 knockout mouse).

2.1.9. Tissue Culture Media.

Media	Components
DMEM	Dulbecco's Modified Eagles Media supplemented with 10% FCS and 2mM L-glutamine
SLM	Special Liquid Media supplemented with 10% FCS and 2mM L-glutamine
RPMI	RPMI Media supplemented with 10% FCS and 2 mM L-glutamine

2.2. Methods.

2.2.1. Cell Maintenance

The medium requirements of the cell lines (and clones) used throughout this are listed in section 2.2.3. Antibiotics and antifungal agents were not used unless otherwise stated in the text or figure legends.

All cell lines were grown in 90mm tissue culture plates in a 5% CO_2 atmosphere at 37°C. Cells were routinely subcultured every 2 to 4 days, unless otherwise stated. All cell culture was carried out in a category III laminar-flow-hood. All cell lines were tested for mycoplasma on a routine basis, and were always negative.

2.2.2. Determination of viable cell numbers

Viability of cultured cells was determined by the trypan blue dye exclusion assay. Following trypsinisation, 50µl of cell suspension was mixed with an equal volume of 0.05% (w/v) trypan blue. Viable cells can be counted using a Neubauer haemocytometer on the basis that non viable cells lose membrane integrity and the blue dye enters them. Cell number per ml of suspension was determined by multiplying number of live cells from 2 x 16 square grids, by 10^4.

2.2.3. Transfection of cell lines

The most commonly used technique for introducing foreign DNA into cells in this thesis was by the calcium phosphate method, and is described below. It should be noted that for transfection by chemical means DNA was prepared using Qiagen Maxi plasmid preparation kit, section 2.5.3.

Cells were plated at a density of 1 x 10^5 to 1 x 10^7 cells in a 90mm petri dish, depending on cell growth rate, and allowed to grow for 16 hr at 37°C in an atmosphere of 5% CO_2. Following this time, fresh full media was added to the plates.

To prepare the DNA precipitate, 20μg of appropriate plasmid DNA was mixed with 62.5μl of 2M calcium chloride solution and sterile water added to a total volume of 500μl in an eppendorf tube. To a second eppendorf 500μl of 2 x HBS (38mM Hepes, 274mM NaCl, 74mM KCl, 1.6mM Na_2HPO_4-H_2O, 11mM glucose, pH 7.05; filter sterilised) was added. The DNA containing solution was then added dropwise to the 2 x HBS, whilst bubbling air through the HBS. This enhances the formation of a fine precipitate. The precipitate was then allowed to form for 30 min. at room temperature.

The precipitate was resuspended and added directly to the medium of the monolayer. Cells were incubated for 5 hr at 37°C in an atmosphere of 5% CO_2.

After this time cells were washed with PBS, and then a solution of 15% glycerol in HBS was added and cells were left at room temperature for exactly 2 min. This enhances the uptake of the calcium phosphate-DNA precipitate. The cells were washed twice more with PBS, then full medium added and the cells left to grow for 24 to 48 hr.

For selection of stably transfected cells, the cultures were trypsinised and 5 to 10% of the cell suspension transferred to each of 10 petri dishes containing 10ml of appropriate selection media, as described in table 2.1.

Colonies were allowed to form over a period of 1 to 4 weeks, depending on cell growth rate, and selection media replaced every 3 to 4 days. After this time resistant colonies were ring cloned (see below).

Cell Line	Medium for w.t. cells	Transfected gene (plasmid) and drug selection.
CarB	SLM	tk (pGK-tk) or E-Cadherin* or Cx43 antisense (pRc/CMV-Cx43 AS) or β-gal (pSVL-gal). Selection, 400μg/ml G418. E-Cadherin*, then tk (pCEP4-tk), separate transfections. Dual selection, 400μg/ml G418 and 300μg/ml hygromycin, respectively. tk (pGK-tk), then tp*, separate transfections. Dual selection, 400μg/ml G418 and 1.5μg/ml puromycin, respectively.
L929	SLM	tk (pGK-tk) or cd (pcDNA3.1⁻-cd) or tp (pcDNA 1-tp) or β-gal (pSVL-gal). Selection, 800μg/ml G418.
BHK	DMEM	tk (pGK-tk) or cd (pcDNA3.1⁻-cd) or cdtk fusion (pcDNA3.1⁻-cdtk). Selection, 800μg/ml G418. Cx43 antisense (pRc/CMV-Cx43 AS) or β-gal (pSVL-gal). Selection, 800μg/ml G418. tk (pGK-tk), then tp (pCEP4-tp) separate transfections. Dual selection, 800μg/ml G418 and 150μg/ml hygromycin, respectively.
B9	SLM	tk (pGK-tk). Selection, 400μg/ml G418.
MCF-7	RPMI	tk (pGK-tk). Selection, 300μg/ml G418.
B95 (p53⁻)	SLM	tk (pCEP4-tk). Selection, 50μg/ml Hygromycin.

Table 2.1. Transfecion of cell lines. E-cadherin was transfected prior to this thesis (Holden et al, 1997). tp was introduced to CarB tk^+ transfected cells by retroviral transduction, by Adam Patterson, MRC radiation unit, Oxford.

2.2.4. Ring cloning of resistant colonies

The position of colonies resistant to drug selection was marked on the bottom of tissue culture plates. Media was aspirated and the cells washed with PBS. Autoclaved metal cloning rings (Gibco BRL) were dipped into sterile vacuum grease and placed over the marked colonies. Approximately 100μl of trypsin was added to each ring until cells had detached and the cells were transferred to 1 ml of selection media in a 24 well plate.

Once cells were at least 20% confluent they were washed in PBS and released with trypsin. Equal numbers of cells were transferred to two wells of a 24 well plate. Cells in one well were allowed to grow in normal media while cells in the second well were tested for expression of the transfected gene. To test for expression of the suicide gene (tk^+, tp^+ or cd^+) cells were incubated for 5 days with the corresponding prodrugs, GCV (5μM), DFUR (2μM) or 5FC (1000μM), respectively.

To test for expression of the β-gal lineage marker, cells were examined for the ability to convert the chromagenic substrate x-gal to a blue product, section 2.2.13.

Clones expressing Cx43 AS were analysed by Western Blot analysis, section 2.4.2.

2.2.5. Population doubling time

10^5-10^7 cells were plated in 35 mm dishes and allowed to settle for 8 hr. After this time, and every 24 hr for 2 days, number of live cells was determined as described above. From growth curves, population doubling times were determined.

2.2.6. Frozen cell stocks

Aliquots of wild type and transfected cell lines were maintained in liquid nitrogen for long term storage. To freeze cells, 10^6-10^7 cells were washed in PBS, trypsinised and 10 ml of

media added (to inhibit the trypsin). Cells were pelleted by centrifugation at 1000 rpm for 5 min.. The pellet was resupended in 1 ml appropriate media supplemented with 10% Dimethylsulphoxide (DMSO). The suspension was transferred to a 1ml Nunc cryotube, wrapped in cotton wool and stored at -70°C overnight, to facilitate a slow cooling process. The following day vials were transferred to liquid nitrogen vats.

2.2.7. Determination of cell survival

Transfer of toxicity in enzyme prodrug systems may require cell to cell contact. Therefore, cells were seeded to achieve approximately 25% confluence at the time of prodrug treatment. At a later time in the assay, when cells were confluent, transfer to 96 well plates was performed to enable further cell growth and then cell survival to be assessed .

Monolayers of tissue culture cells were trypsinised and viable cells counted using the trypan blue dye exclusion method (section 2.2.2.). Mixed cultures of cells, (0.5 to 2.5 x 10^5 viable cells) with and without suicide gene, were seeded, in triplicate, in 1 ml total volume and allowed to settle overnight (see text and figure legends for details). After this time most cells were in contact with the surrounding cells.

Prodrug was dissolved in PBS to provide a stock solution. At time of use, this was diluted so that 12.5µl could be added to each well to provide the correct concentration (see figures). Controls were treated with PBS. Cells were then allowed to grow for a further 48 hr.

Transfer of cells to 96 well plates was used to prevent over growth of cells which would lead to cell death in controls and distort results. This cell transfer was termed 'standard protocol' and is described below. It is important to stress that in all assays prodrug concentration was the same before and after cell transfer. The exceptions were medium transfer and filter separation experiments (section 2.2.9.) where the medium in 96 well plates did not contain prodrug.

Using a multichannel pipette (costar, 8 channel) 120µl of media were placed in each well of a 96 well plate and diluted prodrug added at the required concentrations (where required). Cultures from the 24 well plates were washed in PBS and trypsinised with 5% trypsin in P.E. and 10µl of the cell suspension was transferred from each well of the 24 well plate to four corresponding wells of a 96 well plate, so that prodrug concentration throughout each individual experiment was constant. Cells were allowed to grow for a further 72 hr.

At this time cell survival was quantitated using the cell proliferation assay (see below).

2.2.7.1. Cell proliferation assay

Cell proliferation was measured by using the Promega Cell Titre 96^{TM} Non-Radioactive Cell Proliferation Assay. This is an indirect method for determining cell number achieving comparable results to ^{3}H thymidine incorporation. The assay is based on cellular conversion of a tetrazolium salt (MTT) into a coloured formazan product which can be detected spectrophometrically by an ELISA plate reader. The protocol below is based on that described by Promega.

To assay cell survival 14µl of the dye solution (MTT based) was added to each well of a 96 well plate and colour developed by incubating plates at 37°C for 1 to 4 hr. Time of incubation depended on cell density and the metabolic activity of a particular cell line to convert MTT to formazan product.

The reaction was terminated by the addition of 100µl of stop solution per well and formazan crystals were solubilised by incubation at 37°C overnight.

Absorbance was determined spectrophotometrically at dual wavelengths of 590nm and 630nm using an ELISA plate reader (Dynatech) with shaking facility. By using dual

wavelengths greater accuracy is achieved as interference through plate damage, such as scratching, is reduced.

Survival was calculated by comparing absorbance of treated versus untreated cultures.

2.2.8. GJIC in tp / DFUR bystander effects

Cultures were seeded at high or low density were used to assess whether the presence of gap junctions enhances the bystander effects in the tp system.

Mixed cultures of tp^+ with tp^- cells were seeded at 5-15 x 10^4 cells / ml for high density and 1-3 x 10^3 cells / ml for low density experiments. Cells were allowed to settle overnight. Application of prodrug, transfer to 96 well plates and determination of cell survival were performed as described previously (standard protocol, section 2.2.7.).

2.2.9. Role of medium mediated toxicity in bystander effects

The mechanism of transfer of toxic metabolites from cells transfected with suicide genes ($gene^+$) to those with out the gene ($gene^-$) was investigated by medium transfer and filter separation experiments.

For medium transfer, $gene^+$ cells were seeded at 5-10 x 10^4/ml and $gene^-$ cells were seeded at 5-10 x 10^3/ml in 24 well plates. Cells were allowed to settle for 24 hr.

The appropriate concentration of prodrug (see text and figure legends for details) was added to $gene^+$ cells and incubated at 37°C for 48 hr.

After this time, media from the $gene^-$ cells was replaced with the drug treated media from the $gene^+$ cells. Cells lacking the gene were then incubated for a further 48 hr.

Cells were then transferred to 96 well plates (section 2.2.7.) and survival assayed (section 2.2.7.1.).

Two negative controls were also included. Media with prodrug only (no gene$^+$ cells) was incubated for 48 hr prior to transfer to gene$^-$ cells. In the second control, gene$^+$ cells were incubated with media (no prodrug) before transfer.

For filter experiments, 5-15 x 10^4 gene$^-$ cells were seeded in 24 well plates in 0.5ml (see text and figure legends for details). Using sterile forceps, 0.02μm filter inserts were then placed into the wells containing the gene$^-$ cells and 5-15 x 10^4 gene$^+$ cells (in 0.5 ml) were seeded in the insert. The appropriate concentration of prodrug was added immediately and cells were incubated at 37°C for 3 days. After this time, gene$^-$ cells were transferred to 96 well plates (section 2.2.7.) and survival assayed (section 2.2.7.1.).

2.2.10. Time course of the tk / GCV bystander effect

The effect of short periods of GCV application on colony formation was used to determine the earliest time at which toxicity is transferred from tk$^+$ to tk$^-$ cells.

5-20 x 10^5 tk$^+$ cells alone or a mixture of tk$^+$ and tk$^-$ cells (1:1) were seeded in 35 mm dishes and allowed to settle for 4 hr.

20μM GCV was applied to the media of the cultures and incubated at 37°C for various periods of time.

Cells were washed with PBS, trypsinised and an aliquot of the cell suspension was transferred to a 100mm dish containing full media without prodrug. Cells were incubated until visible colonies had formed and at this time colonies were stained with Giemsa stain, as described in section 2.2.14. Survival of the drug treated cultures was compared to untreated cultures.

2.2.11. Effective range of the tk / GCV bystander effect

A colony based assay was used to determine the approximate number of tk^- cell diameters the tk / GCV bystander effect can pass through. In this experiment tk^- colonies were identified using the β-galactosidase lineage marker.

25 tk^- β-gal^+ cells were plated in each of four 35 mm dishes. Three sets of four plates were seeded for each size of tk^- β-gal^+ colony that was investigated.

Once desired colony size was attained, 1-3 x 10^5 tk^+ β-gal^- cells were added to two plates of each set and allowed to settle for 24 hr (see table below).

At this time 1 set of plates was fixed and stained with x-gal (section 2.2.13.) to keep a record of initial colony size.

20μM GCV was applied to two wells in each set, one well containing only tk^- β-gal^+ and the other containing tk^- β-gal^+ and tk^+ β-gal^- (see table below). Cells were incubated for 2 days at 37°C. A second set of plates were fixed and stained with x-gal for final colony size.

The cells in the final set of plates were trypsinised and aliquots from each plate in the set used to assess the ability of the cells to form colonies.

Plate Number	Purpose	Cell types in dish	2 days 20μM GCV treatment (sets 2 & 3)
1	Control	tk^- β-gal^+	No
2	Control	tk^- β-gal^+	Yes
3	Control	tk^- β-gal^+ and tk^+ β-gal^-	No
4	Test	tk^- β-gal^+ and tk^+ β-gal^-	Yes

2.2.12. Measurement of gap junctional intercellular communication by dye transfer

The level of homologous communication was measured in wild type and transfected cells so that the effect of increasing levels of GJIC on the tk / GCV bystander effect could be determined, and to ensure that transfection of suicide genes did not affect communication. Measurement of GJIC involved micro-injecting cells with Lucifer Yellow CH tracer dye, based on the method described by Pitts and Kam (1985).

To prepare for the assay, cells were grown until 80% confluent in 60 mm Falcon tissue culture plates. In addition, micro electrodes were made from 1.0mm OD "Kwik-fill" thin-wall glass capillaries and back-filled with a 4% aqueous solution of Lucifer Yellow CH.

Immediately before dye injection, media was replaced with fresh media buffered with 25mM Hepes (in place of sodium bicarbonate). Cells were transferred to the heated (37°C) stage of a Leitz Diavert inverted microscope and an individual cell, with apparently normal morphology, was selected and iontophoretically injected with dye using the micro electrodes prepared previously. Injections were performed for 2 min. using a current of 10nA in 0.5 second pulses at 1 Hz and the process monitored with UV (epi-illumination) or visible (phase contrast) light sources. The extent of dye spread to neighbouring cells was recorded immediately and every fifth injection photographed.

2.2.13. β-galactosidase staining

Cells expressing the β-galactosidase lineage marker can be identified through a colorimetric assay utilising 5-Bromo-4-Chloro-3-Indyol β-D-Galactopyranoside (x-gal).

Monolayers of cells were washed once in PBS and fixing solution added (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 30 min.. Following this time, the cultures were washed again with PBS and incubated with staining solution for 8 hr. Staining solution was prepared by combining the following volumes of stock solutions:-

250µl of x-gal (40mg / ml) in DMSO

100µl of 0.5M Potassium Ferricyanide ($K_3Fe[CN_6]$)

100µl of 0.5M Potassium Ferrocyanide ($K_4Fe\ [CN_6]\cdot H_2O$

20µl of 1M $MgCl_2\cdot H_2O$.

After this time the cultures were washed three times in water, inverted and allowed to air dry.

2.2.14. Giemsa staining

Giemsa is a purple stain that binds to cytosolic proteins and can be used for the visualisation of cultured cells.

Cells were washed once with PBS and fixed in methanol for 30 min. at room temperature. Plates were washed with water and 10 ml of Giemsa stain (1 in 10 dilution with water) was added and incubated at room temperature for 10-20 min.. Stained cells were washed three times in water, inverted and allowed to air dry.

2.3. *In vivo* analysis of enzyme / prodrug systems

To further investigate bystander effects, a nude mouse model was used. Tumours were induced using tumorigenic cell lines that had previously been investigated in culture systems.

Pilot studies using small numbers of animals were set up initially to determine rate of tumour formation in animals, and to ensure that unnecessary distress was not caused to the animals by tumour burden and prodrug treatment. These studies enabled the following regime to be employed.

Monolayers of gene$^+$ and gene$^-$ cells were trypsinised and cell viability determined by trypan blue dye exclusion. The required number of cells were pelleted, in full medium, by centrifugation at 1000 rpm for 5 min., and the pellet resuspended in serum free medium to give

a cell suspension containing 10^6 cells / ml gene $^+$ and gene $^-$ cell suspensions were mixed at specific ratios (see figures legends and text).

Tumours were generated by injecting 100µl of cell suspension, subcutaneously, into each flank of nude mice with a 1ml syringe.

Tumours were allowed to form for approximately 4 (CarB) or 10 (BHK and L929) days at which time intraperitoneal administration of prodrug was initiated and continued for 7 or 14 days (see text and figure legends). Solutions of prodrug were prepared and injected as follows:-

- GCV : Stock solution 11mg / ml PBS. 4µl / gram body weight injected (=45mg / kg).
- FC : Stock solution 12.5mg / ml PBS. 20µl / gram body weight injected (=250mg / kg).

During the period of treatment, weight of the animals monitored to calculate appropriate drug administration and to ensure the animals remained healthy. Tumours were measured in two dimensions during and after treatment.

Animals were removed from the study when the tumour burden exceeded $500mm^3$ or if animals became sick or if the experiment continued for greater than 6 months.

Animals were terminated by placing them in an environment containing high levels of CO_2, (schedule 1 procedure) and tumours removed. Samples were initially prepared for histological or enzymic analysis by snap freezing. Alternatively, tissue was digested (10% trypsin, in P.E. for 1 hr, agitating) and released cells grown under tissue culture conditions for subsequent analysis. Growth medium contained the fungicide, amphotericin B, and antibiotic, gentamycin, for 7 days following tumour removal from mice.

2.4.1. Protein calibration

For Western blot and tk enzyme activity analysis quantitation of protein levels was required. This was performed using the Pierce Micro BCA Protein Assay Reagent kit. The protocol is outlined below:-

For 10mls of working solution 4.8ml and 0.2ml of solutions MB and MC are mixed, then 5mls of solution MA is added and mixed.

2μl of sample was diluted into 1ml water, before adding 1ml of working solution. In the same way, standards containing 0, 5, 10, 15 or 20μg/ml BSA were prepared. Tubes were mixed prior to incubation for 60 min. at 60°C.

After this time, samples were then cooled to room temperature and absorbance of standards and samples analysed spectrophotometrically at 562nm.

A curve of the standards was plotted and protein concentration of the samples determined.

2.4.2. Western Blot analysis

Western Blot analysis was used to examine alterations in the level of connexin 43 (Cx43) expression in wild type and transfected (stable transfection expressing Cx43 AS) cell lines. Clones that were down regulated in gap junctional intercellular communication could be used to further investigate the tk / GCV bystander effect.

Cell lysates were prepared from 80% confluent monolayers in 35mm tissue culture plates. Monolayers were washed with PBS and 300μl of cell lysis buffer added (150mM NaCl, 20mM tris, 20mM sodium pyrophosphate, 10μM $NaVO_4$, 2mM PMSF, 0.001% aprotinin, 1% NP40; pH 6.8). Samples were collected with a cell scraper and transferred to an eppendorf

tube, prior to sonication for 30 seconds. Protein levels were calibrated, using the Pierce BCA kit, (as described in section 2.4.1.) and stored on ice until use.

Proteins were resolved, according to their molecular weight, using sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE). The components required are described im Table 2.2.

To prepare the gel, two 16 x 16 cm glass plates were clamped together and sealed with a 0.75 mm rubber gasket. The resolving gel mix was carefully poured between the plates, in an effort to minimise air bubble formation. The mixture was overlaid with water-saturated isopropanol and allowed to polymerise at room temperature (exposure to air inhibits the reaction). Once the gel had set, isopropanol was removed by capillary action using Whatman 3MM paper and replaced with stacking gel mix. A gel comb was added and the solution allowed to polymerise at room temperature. At this time the gel apparatus was transferred to an electrophoresis tank and the reservoirs filled with 1 x electrophoresis buffer (50mM Tris, 50mM glycine, 0.5% SDS). The comb was removed and the wells washed out with buffer to remove unpolymerised stacking gel.

40μg of protein lysate was added to an equal volume of 2 x loading buffer (5% SDS, 10% glycerol, 80mM Tris, 4% 2-mercaptoethanol, 0.2% bromophenol blue; pH 6.8). Samples were boiled for 1 min., prior to loading and then resolved at a constant current of 30mA / gel for 2 hr.

In preparation for semi-dry blotting of proteins to nitrocellulose, six sheets of Whatman 3MM filter paper and one sheet of nitrocellulose ECL-hybond membrane were cut to match the dimensions of the gel, and prewet in transfer buffer (0.5M tris, 40mM glycine, 0.04% SDS, 20% methanol).

Once electrophoresis was completed, the gel apparatus was dismantled, the gel trimmed and transferred onto the nitrocellulose filter, cutting one corner for orientation.

10% Resolving gel	30% Acrylamide / 0.8% Bisacrylamide	8 ml
	10% sodium dodecyl sulphate	0.23 ml
	1.5 M Tris-HCl pH 8.8	5.8 ml
	water	10 ml
	10% Ammonium persulphate (freshly made)	0.2 ml
	TEMED	10 µl
5% Stacking gel	30% Acrylamide / 0.8% Bisacrylamide	1 ml
	10% sodium dodecyl sulphate	0.11 ml
	1 M Tris-HCl pH 6.8	1.4 ml
	water	3.5 ml
	10% Ammonium persulphate (APS)	50 µl
	TEMED	2.5 µl

Table 2.2. Separation of proteins using SDS-page

Three sheets of prewet 3MM paper were placed on the lower graphite plate of a semi-dry electroblotter. The gel and membrane were positioned, with gel upper most, on the filters, and three more sheets of prewet 3MM filter paper were added. The upper graphite plate was replaced and electroblotting performed at a current of 200mA for 1 hr.

On completion of transfer, the nitrocellulose membrane was shaken in blocking solution (4% Marvel in PBS) for 3 hr at room temperature.

Primary antibody to connexin 43 (Zymega laboratories) was diluted 1:1000 in blocking solution and applied to the membrane and shaken gently for 3 hr at room temperature.

The membrane was washed 4 times (1, 15, 10 and 5 min. / wash) in blocking solution.

Secondary antibody was diluted 1:1000 in blocking solution and placed on the membrane for 1 hr at room temperature. This was followed by 3 washes in blocking solution (1 x 1 min. and 2 x 45 min.), one 5 min. wash in TBS-Tween (25mM tris, 150mM NaCl, 0.05% Tween-20; pH 7.5), and by two twenty min. washes in blocking solution.

To determine the position of antibody binding and hence connexin 43, an Amersham Enhanced chemilluminescence (ECL) kit was used, in accordance with the manufacturers instructions. Equal volumes of solutions 1 and 2 were mixed in a universal container and applied to the nitrocellulose membrane, with gentle agitation, for 1 min. After this time the excess solution was discarded and the membrane wrapped in saran-wrap, before exposure to Kodak XAR-5 autoradiographic film for 60 min.

2.4.3. tk enzyme assay

To prepare lysates for the enzyme assay, a subconfluent plate of cells were trypsinised and pelleted by centrifugation (1000rpm / 5min). The cells were washed by resuspending in PBS, then collected by centrifugation as before. This was repeated once. The assay is based on that described by Brinster et al, (1981). The pellet was then resuspended in 400µl

homogenisation buffer (10mM KCl, 2mM $MgCl_2$, 10mM Tris-HCl, 1mM ATP, 10mM NaF, 50mM ε-amino caproic acid, pH 7.4) and subjected to 3 rounds of freeze / thaw (liquid nitrogen / 37°C) treatment. Cell debris was removed by centrifugation (14000 rpm / 15 min. / 4°C) and protein levels of lysates determined (section 2.4.1.). 2μg protein was added to 28μl reaction buffer (10mM $MgCl_2$, 25mM NaF, 10mM β-mercaptoethanol, 56μM tetrahydrouridine, 150mM Tris-HCl, 10mM ATP, 2μl (1μCi) of ^{125}I Iododeoxycytidine, pH 7.5) and reactions were incubated for 90 min. at 37°C.

At this time, samples were spotted to DE81 Whatman ion exchange filters and air dried.

To remove unincorporated ^{125}I Iododeoxycytidine the filters were washed (20 min. per wash) as follows:-

4 times in 3mM ammonium formate
Once in water
Once in methanol.

Filters were air dried, placed in scintillation fluid and radioactivity was measured in a Beckman LS5000CE scintillation counter. Picomoles of iododeoxycytidine phosphorylated per hr was calculated, and specific activity per mg protein determined.

2.4.4. cd enzyme assay

Cells were trypsinised and washed by the same process as described in section 2.4.3. The assay was based on that described by Kanai et al, (1997). The pellet was resuspended in 400μl of reaction buffer (100mM Tris-HCl, 1mM EDTA, pH7.8) and cells were lysed by 3 rounds of freeze / thaw (liquid nitrogen / 37°C) treatment. Cell debris was removed by centrifugation (14000 rpm / 15 min. / 4°C) and protein levels of lysates determined (section

2.4.1.). 10μg protein was diluted in a total of 15μl reaction buffer (containing 2μl of 0.14mCi/mmol) and reactions were incubated for 5 min. at 37°C. At this time the reaction was terminated by the addition of 345μl 1M acetic acid.

1ml SCX bond elute columns were pre-equilibrated with 500μl 1M acetic acid by centrifugation (2000rpm / 2min). Reaction samples were loaded to column and centrifuged (2000rpm / 2min). The ^{3}H uracil produced was eluted with 1 ml 1m acetic acid and radioactivity was measured in a Beckman LS5000CE scintillation counter. Picomoles of cytosine converted to uracil per min. was calculated, and specific activity per mg protein determined.

2.5. Nucleic Acid Techniques

2.5.1. Bacterial Transformation

The competent bacterial strain DH5α was purchased from Gibco BRL in 200μl volumes. Cells were allowed to thaw on ice, aliquoted in 30μl volumes and frozen rapidly on dry ice until further use.

For transformation 30μl of competent cells were thawed on ice and between 1 and 50ng of DNA added and cells incubated on ice for 30 min.

The cells were then heat shocked for exactly 45 seconds and incubated on ice for 2 min. 170μl of SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 9mM NaCl, 2,5mM KCl, 10mM $MgCl_2$, 20mM glucose) was added and cells shaken at 225rpm at 37°C for 1 hr to promote expression of the ampicillin resistance marker.

After this time transformed cells were spread onto agar plates (1.5% in L-Broth) supplemented with ampicillin (50mg/ml) and incubated up-side-down at 37°C overnight to allow colony formation.

Sterile yellow pipette tips were used to transfer colonies into plastic universals containing 4ml L-Broth supplemented with 50mg/ml ampicillin and the culture expanded by shaking (225 rpm) for 6 hr at 37°C. After this time mini or maxi plasmid preps protocols were followed.

2.5.2. Mini plasmid preparations

Depending on the quality and quantity of mini prep DNA required, one of two procedures was followed. The first yields crude plasmid DNA which can be used for restriction mapping only and is based on the Qiagen maxi prep kit, while the second produces a larger quantity of high quality plasmid DNA, requiring the Nucleon mini prep kit.

For both preparations, 1.5ml of bacterial culture was pelleted by centrifugation at 14000rpm for 30 seconds. The supernatant was discarded and samples were vortexed after the addition of each of the buffers 1, 2 and 3 (supplied with kits) from corresponding Qiagen (300µl of each buffer) or Nucleon kits (150µl each buffer). Cell debris was pellet by centrifugation at 14000rpm for 5 min. The remainder of each protocol is described below.

For crude plasmid DNA (Qiagen):

Supernatant was transferred to a fresh tube containing 700µl propan-2-ol and DNA was precipitated by vortexing, then pelleted by centrifugation at 14000rpm for 10 min. The pellet was washed and dried as described in section 2.5.8.

Nucleon Mini-prep:

Supernatant was transferred to spin column containing 200µl of DNA binding resin and centrifuged for 30 secs at 14000 rpm. The effluent was discarded and 500µl of 1x wash buffer added to the sample, before centrifugation for 2 min. at 14000rpm. The resin was allowed to dry for 5 min. at room temperature before eluting the plasmid DNA in 50µl of elution buffer, by centrifugation for 1 min. at 14000rpm.

2.5.3. Maxi plasmid preparations (Qiagen kit)

The bacterial suspension prepared in section 2.5.1. was transferred to a flask containing 250 ml of L-Broth supplemented with 50µg / ml ampicillin and the culture expanded by shaking (225 rpm) over night at 37°C.

The bacteria were pelleted by centrifugation at 5000rpm for 10 min. in a Sorval centrifuge (JLA 10500 rotor) and the supernatant discarded.

The bacterial pellet was resuspended in 4 ml buffer P1 (50mM tris, 10mM EDTA, 100µg/ml RNAse, pH 8.0) and bacteria were lysed by the addition of 4 ml buffer P2 (0.2M NaOH, 1% SDS (w/v)). 4 ml buffer P3 (2.55M potassium acetate, pH 8.4) was added and the sample incubated at room temperature for 10 min. to precipitate protein. The sample was transferred to a Qiagen filter tip, and incubated at room temperature until precipitated matter had formed the upper layer of the sample. The soluble fraction was collected, in a beaker, by plunging the sample through the filter.

A Qiagen-500 tip was equilibrated with 5 ml of buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol, pH 7.0) and the soluble material from above applied to the tip. The sample was then washed twice with buffer QC (1M NaCl, 50mM MOPS, 15% ethanol, pH 7.0) and eluted with 5 ml buffer QF (1.25 M NaCl, 50mM MOPS, 15% ethanol, pH 8.2).

DNA was precipitated, by mixing vigorously with 0.7 volumes (3.5 ml) isopropanol, and pelleted by centrifugation at 10000 rpm at 4°C for 30 min. in a Beckman centrifuge (JA 25.50). The pellet was washed with 70% ethanol, resuspended in 300µl water and the concentration of nucleic acid determined.

2.5.4. Determination of nucleic acid concentration

The concentration of nucleic acids was determined spectrophotometrically. The spectrophotometer (Beckman) was calibrated using a water blank, then the absorbance of samples diluted in water was measured at wavelengths of 260 and 280 nm in a quartz cuvette with a path length of 1 cm. For plasmid and genomic DNA an A260 of 1 was taken to correspond to 50mg/ml and for primers an A260 of 1 was taken to correspond to 33mg/ml.

2.5.5. Polymerase Chain Reaction (PCR)

PCR amplification of the cd and tk cDNAs was used for two purposes:-

1) Subcloning of the suicide genes into alternative expression vectors when appropriate restriction sites were not available.

2) The addition of genetic material to the ends of the cDNAs. This included addition of Kozak sequence (to enhance translation) and restriction sites (for the generation of cd-tk chimeras).

Amplification of the tk gene was performed using the proof reading DNA polymerase, pfu, while amplification of the cd gene was accomplished with a Boehringer Mannheim PCR kit comprising a Taq / Pwo DNA polymerase mix. Components of each reaction are described in table 2.3.A., primer pairs in table 2.3.B., primer sequence table 2.3.D., and cycling conditions detailed in table 2.3.C. PCR amplification was performed using a Perkin Elmer (Cetus 480).

Table 2.3.A. - PCR reaction components	Volumes of components	
Components of reaction	**tk / pfu reaction**	**cd / Taq-Pwo reaction**
Water	49 µl	30.25 µl
10 x Buffer	10 µl (pfu buffer)*	5 µl (Buffer 1) *
DMSO	10 µl	-
50% Glycerol	10 µl	-
2.5mM dNTPs (dCTP, dGTP, dTTP, dATP)	8 µl	7 µl
5' Primer (20 µM)	5 µl	2.5 µl
3' Primer (20 µM)	5 µl	2.5 µl
Template (1ng / µl)	2 µl	1 µl
DNA Polymerase	1 µl	0.75 µl
Total Volume	100 µl	50 µl

* Buffer supplied with enzyme

Table 2.3.B. - Primer pairs

cDNA	Primer pairs [a]	Purpose	PCR program[b]
tk	tk1 and tk2	Amplifies the tk gene	2
cd	cd1 and cd2	Adds Kozak sequence to cd gene	1
tk	tk3 and tkcd1	Adds Kozak sequence and XbaI site for tkcd chimera	3
cd	tkcd2 and cd2	Adds XbaI site for tkcd chimera	1
cd	cd1 and cdtk1	Adds Kozak sequence and XbaI site for cdtk chimera	1
tk	cdtk2 and tk4	Adds XbaI site for cdtk chimera	2

[a] See Table 2.3.B. for details of primer sequences

[b] See table 2.3.C. for details

Table 2.3.C. - Primers for PCR amplification

Primer Name	Primer Sequence 5'→ 3'
tk 1	ATG GCT TCG TAC CCC GGC
tk 2	TCA GTT AGC CTC CCC CAT
tk 3	GAG GAG TCT AGA GCC ACC ATG GCT TCG TAC CCC
tk 4	GTG GTG TCT AGA TCA GTT AGC CTC CCC CAT
cd 1	GCC GCC ACC ATG TCG AAT AAC GCT TTA CAA
cd 2	TCA ACG TTT GTA ATC GAT GGC
tkcd 1	GTG GTG TCT AGA GTT AGC CTC CCC CAT
tkcd 2	GAG GAG TCT AGA ATG TCG AAT AAC GCT
cdtk 1	GTG GTG TCT AGA ACG TTT GTA ATC GAT GGC TTC
cdtk 2	GAG GAG TCT AGA ATG GCT TCG TAC CCC

Table 2.3.D. - Cycling conditions for PCR amplification

Program 1	Program 2	Program 3
94°C for 2min - 1 cycle	94°C for 2min - 1 cycle	94°C for 2min - 1 cycle
94 °C for 10sec)	94 °C for 30sec)	94 °C for 30sec)
54 °C for 30sec) 10 cycles	37 °C for 30sec) 5 cycles	42 °C for 1min) 2 cycles
68 °C for 45sec)	72 °C for 30sec)	72 °C for 45sec)
94 °C for 10sec)	94 °C for 30sec)	94 °C for 30sec)
54 °C for 30sec) 25 cycles	54 °C for 30sec) 30 cycles	54 °C for 45sec) 30 cycles
68 °C for 2min)	72 °C for 30sec)	72 °C for 45sec)
68 °C for 7min - 1 cycle	72 °C for 7min - 1 cycle	72 °C for 7min - 1 cycle

Table 2.3.E. PCR ligation reactions

Components	**Stratagene cloning kit (pfu)**	**Invitrogen cloning kit (Taq / Pwo mix)**
Water	3.5µl	4µl
10 x Ligation Buffer	1µl	1µl
Cloning vector	pCR-Script 1µl (10ng)	pCR™ II vector 2µl (50ng)
Fresh PCR product	2µl	2µl
10mM rATP	0.5µl	-
Srf I enzyme (5 U/µl)	1µl	-
T4 DNA Ligase (4 U/µl)	1µl	1µl
Ligation Time	1 hr at room temperature	4 hr at room temperature

Following amplification, an aliquot of the PCR product was visualised by gel electrophoresis (section 2.5.7.). tk PCR products (generated using pfu enzyme) were cloned with a Stratagene pCR-Script cloning kit, while cd PCR products (amplified with Taq / Pwo enzyme mix) were cloned using an Invitrogen TA cloning kit, in accordance with manufacturers instructions which are summarised below:-

Following the ligation reaction, the plasmids were transformed into bacteria, as described previously (section 2.5.1.).

2.5.6. Restriction Digest Analysis

For the purposes of gene cloning and Southern blot analysis DNA was digested with restriction enzymes.

Reactions were performed at 37°C for 1 hr using restriction enzyme diluted in appropriate 1 x React buffer (Gibco).

2.5.7. Agarose gel electrophoresis

For general DNA analysis a 1% agarose gel was used. To prepare the gel, 1g of agarose was dissolved in 100ml 1 x TBE buffer (5 x TBE is 0.45M Tris, 0.45M boric acid, 10mM EDTA, pH8.0) by microwave heating until boiling. The mixture was allowed to cool to approximately 50°C before pouring into the gel cast. The gel was allowed to set at 4°C, after which the combs were removed and the gel was placed in a gel tank containing 1 x TBE with ethidium bromide to a concentration 0.25μg/ml.

DNA samples and molecular weight standards (marker VII: 350bp to 8500bp, Boehringer Mannheim) were mixed with loading buffer ($^1/_6$ volume), loaded and resolved by electrophoresis at 100 volts for approximately 1 hr.

Following this time the gel was illuminated on a 312nm transilluminator and the sizes of DNA fragments compared to the molecular weight standards. A photographic record of the gel was taken on an Appligene imager.

2.5.8. Ethanol Precipitation

In order concentrate or purify aqueous DNA samples, ethanol precipitation was used. The method is a modification of that described by Maniatis et al (1989).

$^1/_{10}$ volume of 3M sodium acetate (pH5) and 2.5 volumes of absolute ethanol were added to the DNA solution. This was mixed well and stored on dry ice for 15 min. Precipitated DNA was pelleted by centrifugation at 15000rpm for 15 min.

At this time supernatant was removed and the pellet was washed in 70% ethanol. The pellet was collected by centrifugation (15000rpm for 5 min.), dried and resuspended in an appropriate volume of water.

2.5.9. DNA Sequencing

DNA sequencing was performed using the dye terminator method on the ABI373 automatic sequencer. 1 μg of plasmid DNA was mixed with 3.2 pmoles of appropriate primer in a total volume of 12 μl in ABI PCR tubes. 8 μl of reaction premix (supplied) was added. PCR was performed on samples as follows:-

Preheat to 96°C

96 °C x 15 seconds)
50 °C x 1 second) 25 cycles
60 °C x 4 min.)

Soak 4 °C

On completion of the reaction, the DNA was ethanol precipitated (section 2.5.8.). Samples then processed by R. McFalane, using Long Ranger gel mix (ABI).

2.5.10. Genomic DNA Isolation

Puregene genomic DNA isolation kit was used to isolate DNA. This protocol uses high salt concentration to precipitate DNA. Prior to DNA isolation, cultured cells were washed in PBS, trypsinised (10% in PE) and transferred to an eppendorf tube for centrifugation (3000 rpm, 1 min.). Alternatively, tumour samples were ground to a powder, in liquid nitrogen, using a pestle and mortar.

To isolate genomic DNA 1ml of lysis solution was added and mixed by pipetting. Samples were incubated at r.t. for 60 min.

After this time, 4µl of RNAseA solution was added and samples incubated for 6 hr at 37°C.

Samples were placed on ice for 10 min., before adding 200µl of ice cold protein precipitation solution. Precipitation was aided by vigorous vortexing (approx. 40 seconds) and then samples were chilled on ice for a further 15 min., prior to centrifugation (14000 rpm, 5 min.).

Supernatant was transferred to a fresh tube containing 600µl of propan-2-ol and DNA precipitated by inversion. DNA was collected by centrifugation, (14000rpm, 30 seconds) washed in 70% ethanol and then repelleted (14000rpm, 10 min.).

After removing supernatant, samples were air dried, resuspended in 50-100µl DNA hydration solution (overnight at r.t.) and stored at 4°C until further use.

2.5.11. Southern Blot Analysis

Detection of the tk gene from tumours containing various proportions of tk transfected cells was performed by Southern blot analysis.

Genomic DNA was isolated by the Puregene DNA extraction method (section 2.5.10.) and DNA concentration determined spectrophotometrically (section 2.5.4.).

15μg of genomic DNA was digested with Sst1 and Sma1 restriction enzymes in a total volume of 30μl, as described in section 2.5.6.

DNA was separated using 1% agarose gel electrophoresis at 100 volts for 3 hr in 1 x TBE buffer.

Following this time the gel was placed in depurination solution (25ml concentrated HCl in 975 dH_2O) and shaken gently for 15 min. at r.t. The gel was then transferred to alkali transfer solution (0.4M NaOH) and shaken for a further 15 min. at r.t.

DNA was then transferred to a nylon membrane (Hybond N^+) by overnight capillary blotting (described by Sambrook et al, 1989) using 0.4M NaOH.

DNA was UV cross linked to the filter using a UV Statalinker 1800 and the positions of the molecular weight standards marked. Hybridisation was carried out as described in section 2.5.11.2.

2.5.11.1. Radiolabelling of cDNA probes

For Southern blot analysis radiolabelled cDNA was produced using the random priming technique described in the Pharmacia Oligo-labelling kit, and is summarised below:-

To generate labelled tk probe, 25-50ng tk cDNA in a volume of 34μl of dH_2O was denatured by boiling and then cooled rapidly on ice.

10μl of reaction buffer (supplied) was added along with 5μl of $\alpha[^{32}P]$-dCTP (3000Ci /mmol) and 1μl Klenow fragment. The reaction was incubated at 37°C for 30-60 min.

Following this time, unincorporated nucleotides were separated from probe by centrifugation at 3000rpm for 2 min. through a Sephadex resin column (Pharmacia). Due to

the size of the unincorporated nucleotides, these remain in the column, while the larger labelled probe is collected in a clean eppendorf tube and used for hybridisation.

The probe was denatured by boiling for 2-3 min. and then cooled rapidly on ice until further use.

2.5.11.2. Southern Blots

Prehybridisation of the Hybond N^+ membranes was carried out in Hybaid bottles at 65°C for 4 hr in hybridisation buffer (20mM dextran sulphate, 4x SETS, 10x Denhardts, 0.1% SDS, 1mg/ml type vi RNA from yeast).

Prehybridisation solution was replaced with hybridisation buffer (pre-hyb buffer with probe) containing the appropriate ^{32}P-labelled cDNA probe, and hybridisation allowed at 65°C for 16 hr. After this time, non-specifically bound probe was removed from the filter by washing as follows:-

2 x SSC / 0.1% SDS	2 x 20 min. washes at 65°C
0.2 x SSC / 0.1% SDS	2 x 30 min. washes at 65°C

The filter was then wrapped in Saran wrap and exposed to Kodak XAR-5 film for 24 hr at -70°C, with two intensifying screens. The film was processed through a Kodak processor.

2.6. Apoptosis Assays

For assays investigating the time course of apoptosis and changes in the cell cycle in GCV treated cultures, cells were seeded at density of 5-15 x 10^4 cells / ml. After settling overnight, cells were treated, at the appropriate time, with 20μM GCV for the specified period (see text and figure legends). In each assay, treated cells and controls were analysed at the same time.

2.6.1. In situ cell death assay

An in situ cell death detection kit (supplied by Boehringer Mannheim, section 2.1.2.) was used to analyse the time course of cell death in tk transfected tissue culture cells treated with ganciclovir. The DNA of cells entering apoptosis or necrosis undergo strand cleavage. This kit utilises immunochemical detection of DNA strand breaks, allowing single cells undergoing cell death to be detected.

The procedure was based on the protocol supplied with the kit, and is described below.

Cells were seeded and treated with GCV as described above. Cell fixation was carried out in freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 30 min. at room temperature.

Following this time, cells were permeabilised in 0.2% Triton X-100 (in PBS) for 5 min. at room temperature, then rinsed twice in PBS. Fresh TUNEL reaction was made by mixing 50µl enzyme solution and 450µl Label solution (supplied) and 50µl of this solution was applied to the cells. Samples were covered with coverslips, to prevent excessive evaporation, and incubated for 60 min. at 37°C in a humidified chamber.

After this time the fixed cells were washed 3 times in PBS, mounted in 50% glycerol/ PBS and viewed under UV or visible light sources using an Orthoplan fluorescent microscope (Leitz).

Positive and negative controls were also included in each experiment. For these, the above protocol was followed, with the following alterations. For the negative control, cells were incubated with Label solution rather than TUNEL reaction. The positive control was treated with 25µl DNAse I (supplied) for 10 min. at room temperature before washing and applying the TUNEL solution.

2.6.2. DNA Laddering

To determine if apoptosis was occurring in ganciclovir treated cultures, genomic DNA was isolated as described in section 2.5.10. DNA fragmentation was analysed using a modified version of the end labelling technique described by Quarrie et al (1996).

4μg of genomic DNA was incubated with 10μl MgCl2, 1μl Klenow fragment, and 0.1μl $\alpha[^{32}P]$dCTP for 10 min. at room temperature.

30μl of 17mM EDTA were added to stop the reaction and 1μl of glycogen was added to enhance DNA precipitation. Ethanol precipitation was carried out as in section 2.5.8., however 1 volume of 7.5M ammonium acetate was used instead of sodium acetate.

Samples were resuspended in 10μl TE and incorporation of radioactivity into DNA was measured in a scintillation counter (Beckman).

Fragmented DNA was separated by agarose gel electrophoresis, using a 1.8% agarose gel (section 2.5.7.).

DNA was fixed in the gel by placing the gel in 7% TCA for 40minutes. After this time the bromophenol blue in the loading buffer turns yellow in colour.

The gel was then dried by capillary action, for 4 to 6 hr, by gentle compression between 3MM filters.

Once dry, the gel was then sealed in plastic and exposed to Kodak XAR5 autoradiographic film for 2 to 24 hr.

2.6.3. Propidium iodide staining for flow cytometry

Flow cytometry was used to examine the DNA content of wild type cells and tk transfectants after varying periods of GCV treatment. Propidium iodide intercalates with DNA

and fluoresces when excited by 488 nm light. By quantitating the level of fluorescence, DNA content of a cell population can be determined.

After GCV treatment, monolayers were harvested with trypsin. An equal volume of 10% FCS (in PBS) was added and the suspension transferred to a 15ml falcon tube. Cells were pelleted using a bench top centrifuge (1000 rpm for 5 min.), washed with 10ml ice cold PBS and repelleted. Cells were resuspended in 1ml ice cold PBS and fixation achieved by adding 9ml of cold 70% ethanol, then incubating on ice for 1hr.

Fixed cells were pelleted and then resuspended in 2ml staining solution (250μg / ml DNAse free RNAse A, 10μg / ml propidium iodide, 0.2% (v/v) Tween 20 in PBS). Samples were incubated at room temperature for 30 min.

Samples were filtered through 71μm nylon gauze, collected in 6ml falcon tube and stored on ice. DNA content was examined by exciting samples using a 488nm argon laser and emissions collected through a 630 / 22 band pass filter, (FACScan, Becton Dickinson). Duplicate readings from 10000 cells per sample were analysed.

Chapter 3. Results - Analysis of the tk⁻ / GCV system in culture

3.1. Introduction

It is well established that the tk / GCV enzyme / prodrug system can cause cell death in a variety of cell lines in culture and *in vivo* (Moolten et al, 1986; Culver et al, 1992; Vile et al, 1993). A bystander effect associated with this strategy, where cells with and without the tk gene are killed, has also been reported (Culver et al, 1992). There are several mechanisms that could lead to the additional, unexpected, cell death. *In vivo* an immune component appears to be involved in the bystander effect. Regression of experimental tumours is seen more frequently in immune competent, compared to immune deficient, animals (Vile et al, 1994). A role of the immune system is also suggested by experiments based on antibody blocking of T-cells, which reduces the antitumour effect (Consalvo et al, 1995). Damage to the tumour vasculature may also contribute to the bystander effect, causing tumour ischemia as retroviral transduction of tumours, with the tk gene, has been shown to reduce the number of capillaries (Ram et al, 1994).

Another explanation for the bystander effect seen in culture and *in vivo* is transfer of toxicity from tk^+ to tk^- cells. The phenomenon is well documented but the mechanism of transfer of toxicity has been a controversial issue. The suggested pathways include transfer of toxic metabolites via gap junctions (Bi et al, 1993) or transfer of the metabolites, tk enzyme or genetic material coding for the enzyme, by some less well defined mechanism that could involve phagocytosis of apoptotic vesicles released from dying tk^+ cells (Freeman et al, 1993). A soluble cytotoxic factor has also been suggested as a minor contributor to bystander killing in this system (Kuriyama et al, 1995).

A bystander effect is likely to be an essential feature of successful enzyme / prodrug systems for cancer therapy and it is important to understand the mechanism of transfer of toxicity for correct clinical application. For example, if gap junctions are involved, then the tk / GCV system may be restricted to tumours with high levels of gap junctional communication, while the other suggested mechanisms would not necessarily limit the use of this strategy. This chapter describes the experiments which have been performed in culture to determine the mechanism(s) involved.

3.2.1. Selection of cell lines

If GJIC is involved in transfer of toxicity in the tk / GCV system, it is anticipated that cells that do not form gap junctions will show no bystander effect and that increasing communication will produce a stronger bystander killing. Ideally a study of this enzyme / prodrug combination would involve selective modulation of communication within chosen cell lines. This would enable gap junctional communication to be the only variable. However, currently available inhibitors lack specificity and do not completely inhibit communication (see section 3.3.5.). Conversely, expression of genes involved in gap junction formation has not introduced communication into cells that show no detectable coupling (see section 3.3.5). It is however, possible to increase the level of GJIC in lines that are poorly coupled and this approach has been adopted (see below). Due to these limitations in modulating communication, a variety of cell lines with differing in levels of coupling were selected to study the tk / GCV bystander effect. These are described below.

Previous work within the group has demonstrated a reduction of GJIC during mouse skin carcinogenesis (see section 1.12.2.; Holden et al, 1997). From this model B9 squamous carcinoma cells (well coupled) and CarB spindle carcinoma cells (poorly coupled) were

selected. These cell lines represent intermediate and late stages of carcinogenesis, respectively. Loss of E-cadherin, an adhesion protein that can facilitate the formation of gap junctions, is one of the changes that occurs during this progression. Transfection of CarB cells with a functional E-cadherin gene increases coupling by >5 fold, but the morphology of the transfectants (termed CarB-E) appears unaffected. If gap junctional communication is a factor in the tk / GCV bystander effect, then the role of increased coupling can be studied with these cells.

These lines have high or low levels of junctional communication. BHK cells (hamster origin) were selected since GJIC is intermediate to CarB cells and B9 or CarB-E cells. This cell line has been used previously to study metabolic co-operation (Sheridan et al, 1979). Cell lines with no communication were also required in this strategy. L929 mouse fibroblast line and MCF-7 human breast cancer line were selected because the clones we have show no detectable communication.

To summarise, cell lines which lack communication or that are poorly, moderately and well coupled have been selected to study the tk / GCV bystander effect (figures 3.1a. and 3.1b.). If gap junctions do play a role in the bystander effect, then the level of communication required and the effect of increasing GJIC can be established. Since these cell lines vary in origin (human, mouse and hamster) results seen will also determine whether species dependent differences effect bystander killing in the tk / GCV system.

3.2.2. Characterisation of cell lines transfected with the tk gene

The cell lines described above were transfected with the tk gene (section 2.2.3.; figures 3.1.a. and b.). Selection with the appropriate drug (G418 or hygromycin B) resulted in formation of colonies. Clones similar in morphology to parental cells were picked and those

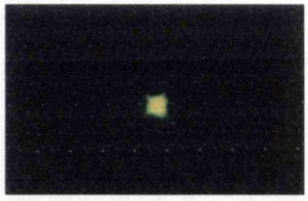
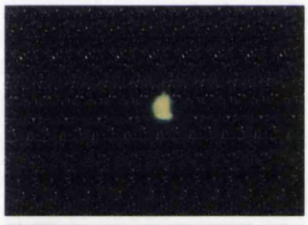
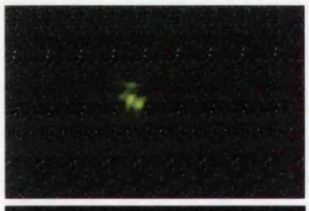
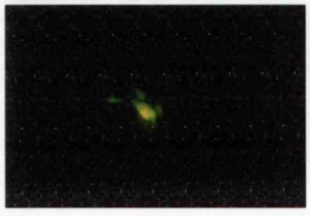
Cell line	Population doubling time (hr)	Gap Junctional Intercellular communication		
		Dye spread (2 min)	Immunofluorescence	Phase
L929	19.1 +/- 1.2	0.0 +/- 0		
L929 tk+	19.6 +/- 1.5	0.0 +/- 0		
MCF-7	48.9 +/- 6.6	0.0 +/- 0		
MCF-7 tk+	43.3 +/- 4.2	0.0 +/- 0		
CarB	22.5 +/- 0.1	3.2 +/- 0.4		
CarB tk+	20.0 +/- 1.5	4.1 +/- 0.7		

Figure 3.1a. Transfection and expression of the tk gene does not significantly alter population doubling time, GJIC or morphology of the cell lines investigated. For determination of population doubling time parental or tk transfected cells were seeded (L929, 1 x 10^5; MCF-7, 2.5 x 10^5; CarB, 1.5 x 10^5 cells / ml), in duplicate, in 35 mm plates and allowed to settle for 24 hr. At this time and then once every 24 hr for 2 days, viable cell numbers were determined using trypan blue dye exclusion. From these data doubling times were calculated. Data represent mean +/- SE of two experiments. GJIC was determined by Lucifer Yellow dye injections as described in section 2.2.12. Data represent a minimum of 10 separate, 2 minute, injections +/- SE. Representative immunofluorescence (L.Y. dye spread) and phase contrast photomicrographs are shown. L929 and MCF-7 cells lack GJIC, whereas CarB cells communicate at a low level.

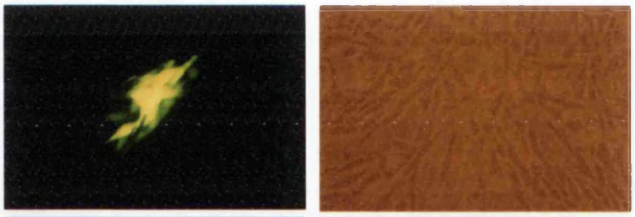
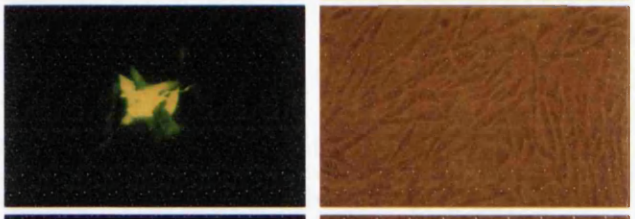
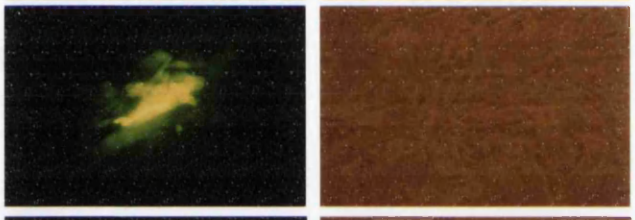
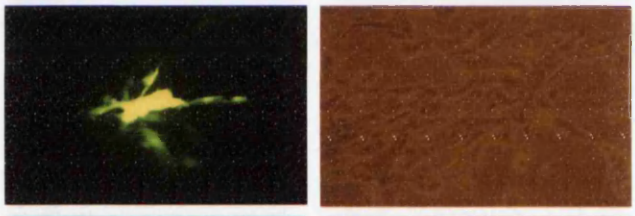
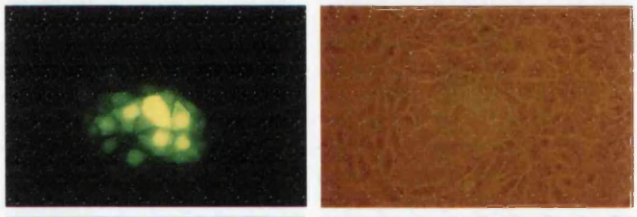
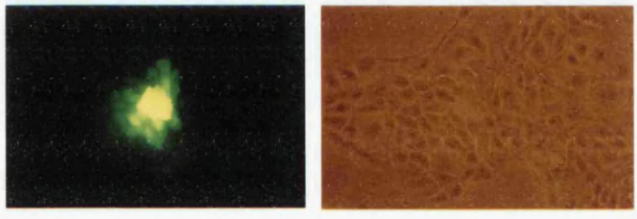
Cell line	Population doubling time (hr)	Gap Junctional Intercellular Communication		
		Dye spread (2 min)	Immunofluorescence	Phase
BHK	10.3 +/- 0.4	9.1 +/- 0.7		
BHK tk+	10.4 +/- 0.1	9.8 +/- 0.9		
CarB-E	16.8 +/- 0.2	25.5 +/- 1.6		
CarB-E tk+	16.4 +/- 0.2	20.2 +/- 1.7		
B9	23.8 +/- 0.9	24.6 +/- 1.5		
B9 tk+	20.4 +/- 0.3	24.1 +/- 2.0		

Figure 3.1b. Transfection and expression of the tk gene does not significantly alter population doubling time, GJIC or morphology of the cell lines investigated. Cells were seeded (BHK, 5 x 10^4; CarB-E, 1.5 x 10^5; B9, 2.5 x 10^5 cells / ml) in 35 mm plates. Population doubling times and GJIC were determined as described in figure 3.1a.

sensitive to 5μM GCV were expanded. It is possible that GJIC of clones could be different to parental cells. If communication is involved in the tk / GCV bystander effect, such a change may effect results. Changes may occur through clonal variation, or integration and / or expression of the tk gene may interfere with gap junction formation, although there are no reports to substantiate the latter suggestion. Therefore the level of coupling of the selected clones was measured by microinjection, for 2 minutes, of the fluorescent dye Lucifer Yellow (section 2.2.12.). This method gives an indication to the extent of communication by counting the number of cells into which dye spreads in a fixed time under standard conditions. Comparison of the results from the tk^+ clones and the parental cells showed that communication is similar (figures 3.1a. and b.). In L929 and MCF-7 cells the dye was restricted to the injected cell, confirming that these cells do not form gap junctions, whereas CarB, BHK, CarB-E and B9 cells showed a progressively increased levels of coupling.

Growth rates may also be important in the effectiveness of killing in the tk / GCV system. GCV kills tk^+ cells that are in S-phase of the cell cycle (Cheng et al, 1983) and cells with a faster growth rate are more susceptible. The population doubling times of clones were measured over 48 hr and were not significantly different (t-test, $p > 0.05$) to those of the parental cells (figures 3.1a. and b.).

This panel of cell lines has been successfully transfected with a functional tk gene and significant differences in morphology, mitotic index or GJIC were not seen between parental cells and the selected clones. This suggests no obvious changes have occurred during the transfection or selection process that might influence the results obtained.

3.3.1. Analysis of the tk / GCV bystander effect in culture

Initial screening showed that clones selected were sensitive to 5μM GCV, a concentration that has no significant effect on survival of parental cells. The following experiments were performed to determine the therapeutic index (the difference in sensitivity between tk^+ and tk^- cells) and to establish whether any of the cell lines selected show a bystander effect. The protocol chosen is summarised below.

Previous reports using the tk system have described the requirement for cell to cell contact and a 5 day treatment regime with GCV to achieve an effective spread of toxicity (Bi et al, 1993; Freeman et al, 1993). For sufficient contact to occur, cell cultures must be at least 10% confluent at the time of GCV treatment. The short doubling times of these cell lines (with the exception of MCF-7) meant that after two days cultures became confluent, and stopped growing, which may not be long enough for maximum cell death to occur at low prodrug concentrations. Growth inhibition could reduce the toxic effect of GCV. To ensure cells remain in cycle during treatment, cells were released with trypsin and aliquots transferred to 96 well plates containing medium (and GCV at the appropriate concentration) so that subconfluence was achieved in untreated controls after a further 3 days. At this time survival was assessed, using the MTT assay (section 2.2.7.1.), and treated cultures compared to untreated (part A, figures 3.2.a. and b.). The use of this protocol enabled treatment of all cell lines, with long or short doubling times, to be performed in the same way. The inclusion of a subculture step means the assay did not just measure survival, but also measured the ability of cells to reattach and grow. It is therefore likely to be more sensitive to toxicity.

In all cell lines studied, there was a reduction in survival when cultures containing 100% tk^+ cells were treated with >0.05μM GCV, whereas parental cells (0% tk^+) were largely unaffected by concentrations below 10μM. A reduction (≈15%) in survival of wild type L929

and MCF-7 cells was seen with 1-10μM GCV. Variations in sensitivity between cell lines are common in the literature, for example, Chen et al, (1995a) have shown 9L cells are resistant to 50μM GCV, while 1μM leads to approximately 15% inhibition of NIH3T3 cells.

Examining the survival of mixed cultures revealed that cells lacking GJIC did not show a bystander effect, for example, in cultures containing 50% L929 tk^+ or 50% MCF-7 tk^+ cells, 10μM GCV killed 47% and 55% of cells respectively, close to the predicted value of 50%. In contrast, 2.5% tk^+ cells killed between 42% and 65% of the communicating cultures (CarB, BHK, CarB-E and B9) when treated with 10μM GCV (part A, figure 3.2.a. and b.). This represents a 17- to 26-fold increase compared to the predicted level of cell death.

An alternative way to present these data is to plot the concentration of GCV required to reduce cell survival by 50% (IC50) against the percentage of tk^+ cells in the culture (part B figures 3.2.a. and b.). Each data point in B has been calculated from a survival curve shown in A. This method of presentation enables the presence or absence of a bystander effect to be readily determined, even when the effect is weak, which is important in later sections (see 5.2.2.). Bystander killing produces a concave curve. In addition, these data enable the effect to be quantitated by calculating the ratios of IC50 for untransfected cultures and those containing 10% tk^+ or 100% tk^+ cells (figures 3.2a. and b.; Table 3.1.). This allows direct comparison of the bystander effects seen in different cell lines.

The parental cell lines showed a small variation in IC50 (<3 fold variation; table 3.1.). The IC50 in L929 and MCF-7 cultures remains high until >50% of cells are tk^+. This is logical because in a population where less than half the cells are tk^+ and there is no significant bystander effect, only tk^+ cells will be killed by low prodrug concentrations. In contrast, IC50's in communicating cultures were reduced by at least 10 fold by the presence of only 2.5% tk^+ cells. Increasing the percentage of transfectants reduced the IC50 further. All the tk^+

Cell line	L929 tk^+	MCF-7 tk^+	CarB tk^+	BHK tk^+	CarB-E tk^+	B9 tk^+
GJIC	0.0 ± 0	0.0 ± 0	4.1 ± 0.8	9.8 ± 1.0	20.2 ± 1.7	24.1 ± 2.0
Specific enzyme activity	11.7 ± 0.8 (10)	25.0 ± 2.3 (2)	19.9 ± 0.8 (14)	16.2 ± 1.2 (12)	12.7 ± 0.3 (2)	12.5 ± 2.4 (2)
IC50 - 0% tk^+ cells	105 ± 2.5	245 ± 45	270 ± 40	145 ± 15	225 ± 5	205 ± 15
IC50 - 10% tk^+ cells	104 ± 6	230 ± 10	0.87 ± 0.24	0.5 ± 0.2	0.86 ± 0.24	0.75 ± 0.03
IC50 - 100% tk^+ cells	0.029 ± 0	0.12 ± 0.05	0.19 ± 0.03	0.041 ± 0	0.2 ± 0.02	0.26 ± 0.04
Bystander effect $^{(0\%\ IC50)}/_{10\%\ IC50)}$	1.04 ± 0.4	1.06 ± 0.15	351 ± 142	292 ± 42	282 ± 73	273 ± 10
Therapeutic index $^{(0\%\ IC50)}/_{100\%\ IC50)}$	3715 ± 215	2242 ± 536	1492 ± 447	3530 ± 280	1134 ± 89	828 ± 173

Table 3.1. Summary of GJIC, tk enzyme activity, bystander effect and therapeutic index in cell lines used to study the tk / GCV system. GJIC has been determined by Lucifer Yellow dye injections as described in section 2.2.12. Data are from a minimum of 10 separate, 2 minute, injections and represent the mean ± SE. Specific enzyme activity has been determined by a tk specific assay (section 2.4.3.). Activity represents pmoles ^{125}I Iododeoxycytidine converted / hr / mg protein and is stated as mean ± SE. The number of experiments is shown in parentheses. Untransfected cells have tk activities of less than 0.2 pmoles conversion. IC50s have been calculated from the survival curves in figure 3.2. Bystander effect and therapeutic index have been determined from these values, as indicated in the table. Data represent mean ± SEM of two independent experiments each performed in triplicate.

cell lines, with or without communication, cultured alone had low IC50's, (part B, figures 3.2.a. and b.). A summary of these results is provided in Table 3.1. For comparative purposes the bystander effect ($^{IC50\ 0\%\ tk+}$ / $_{IC50\ 10\%\ tk+}$) and therapeutic index ($^{IC50\ 0\%\ tk+}$ / $_{IC50\ 100\%\ tk+}$) are given. Calculation of bystander effect from cultures containing 10% tk^+ cells was arbitrarily chosen, although it does represent the upper level of transfection that might be achieved *in vivo*. These values have been calculated from figures 3.2.a. and b., part B. The results show there was no significant bystander effect when GJIC was undetectable, but in cultures that were coupled by gap junctions, even at a low level, there was extensive bystander killing. This difference was not due to relative insensitivity to toxic metabolites because L929 cells had the largest therapeutic index. However for cell lines that did form gap junctions, there was no correlation between level of GJIC and the observed bystander effect. For example, the bystander effect was greater in CarB cultures than in CarB-E cultures, where transfection of CarB cells with E-cadherin has increased the level of communication >5 fold (Table 3.1; figure 3.1.).

If the tk / GCV bystander effect does involve of GJIC then an increase in killing might be expected in response to increased coupling (Table 3.1.). This was clearly not the case and other factor(s) must have influenced the effect. For example, high levels of tk activity would produce high concentrations of GCV-phosphates, and gap junctions when present, may not be the limiting factor in transfer of toxicity. Under these conditions, local concentrations of GCV in tk^+ cells may be restrictive. Other possibilities are that the conversion of GCV-monophosphate (GCV-MP) to triphoshphate (GCV-TP) in the tk^+ and tk^- cells, or the subsequent incorporation into DNA is rate limiting. If tk activity was low in some of the tk^+ cell lines, then this may account for a reduced bystander effect. Production of limited GCV-TP may successfully kill tk^+ cells, since it is possible that only one, or a very few molecules, could

cause cell death. However, higher concentrations of toxic metabolites would be required to kill tk^+ and tk^- cells.

Absence of GJIC may account for lack of bystander effect in some cell lines, but in view of the lack of correlation between increased coupling and increased bystander killing, attention must be given to other possible explanations. Transfer of toxicity could occur via the medium since it has been reported that a cyclic form of GCV-MP can pass through the cell membrane (Prisbe et al, 1986). Kuriyama et al (1995) have suggested the cellular enzyme responsible for conversion of guanosine monophosphate to guanosine 3',5'-cyclic phosphate may also produce a cyclic form of GCV-MP so the lack of such an enzyme may prevent a bystander effect. Another suggestion for the differences in bystander effect that are seen is the secretion of a cytotoxic cytokine from some, but not other, dying tk^+ cell lines. TNFα appears to be released from only some tumour cell lines and can have inhibitory effects in culture (see section 1.11.5.; Sugarman et al, 1985; Freeman et al, 1995).

Phagocytosis of apoptotic vesicles released from dying cells has also been suggested as a mechanism of transfer of toxicity (Freeman et al, 1993). The arguments for a role of such vesicles in the bystander effect has been provided in section 1.11.2. If apoptosis is not a mechanism of cell death in GCV treated tk^+ cells or occurs only to a limited extent, there may not be sufficient production of vesicles to transfer toxicity. This may explain the absence of a bystander effect in some cell lines.

These possibilities must be investigated in order to conduct a comprehensive study of the tk / GCV bystander effect. The methods and results from these analyses are described below.

3.3.2. Bystander effect in relation to level of tk activity

An HSV-tk specific assay (section 2.4.3.) was used to establish the level of enzyme activity in each of the cell lines (Table 3.1.). All parental cells had a specific activity of <0.2 units (pmoles ^{125}I-iododeoxycytidine converted / hr / mg protein), whereas that of the transfectants ranged from 11.7 (L929 tk^+) to 25.0 (MCF-7 tk^+) units. From these data it is apparent that a lack of bystander effect was not due to low tk activity, since MCF-7 tk^+ cells had the highest activity of the six cell lines, but in the communicating cell lines an increase in tk activity appeared to be associated with a stronger bystander effect. For example, CarB tk^+ cells had the highest activity and strongest bystander effect, while a lower activity in B9 tk^+ cells corresponded to a weaker bystander effect (Table 3.1.).

In order to investigate the relationship between tk activity and bystander effect in this system, BHK cells, which are susceptible to bystander killing (Table 3.1.), were transfected with constructs expressing the tk gene and clones with differing tk activities expanded. Clone 1 has an activity of 1.1 units while clones 2, 3, 4, 5, and 6 have activities of 3.4-, 3.8-, 9.5-, 13.8-, and 18.7-fold higher, respectively. Production of clones 4 (BHK cd^+-tk^+) and 5 (BHK $cdtk^+$) is described in section 6.3. The remaining clones express only the tk gene. The clones were morphologically indistinguishable, but there were variations in GJIC (dye spreads 9.1 to 13.6) and doubling times (9.2 to 12.2 hr) that were significant (t-test: $p < 0.05$; figure 3.3.a.; see below).

Survival of mixed (10% tk^+ : 90% tk^-) and separate cultures of tk^+ and tk^- cells was determined after 5 days of GCV treatment, as described previously (section 3.3.1; Section 2.2.7.). From the IC50s, bystander effect and therapeutic index were calculated for each clone and plotted against relative tk activity (figure 3.3.).

A

Clone	GJIC	Doubling Time	Rel. tk activity	Bystander Effect	Therapeutic Index
1	10.8 +/- 0.7	9.8 +/- 0.7	1	5 +/- 1	1040 +/- 196
2	12.4 +/- 0.8	9.2 +/- 0	3.4	32 +/- 5	3643 +/- 440
3	9.1 +/- 0.6	11.5 +/- 1.0	3.8	158 +/- 39	3069 +/- 271
4	13.2 +/- 2.5	10.5 +/- 0.5	9.4	118 +/- 27	1895 +/- 228
5	13.6 +/- 1.0	12.2 +/- 0.2	13.1	314 +/- 31	2441 +/- 60
6	9.8 +/- 0.9	10.4 +/- 0.1	18.7	292 +/- 53	3530 +/- 258

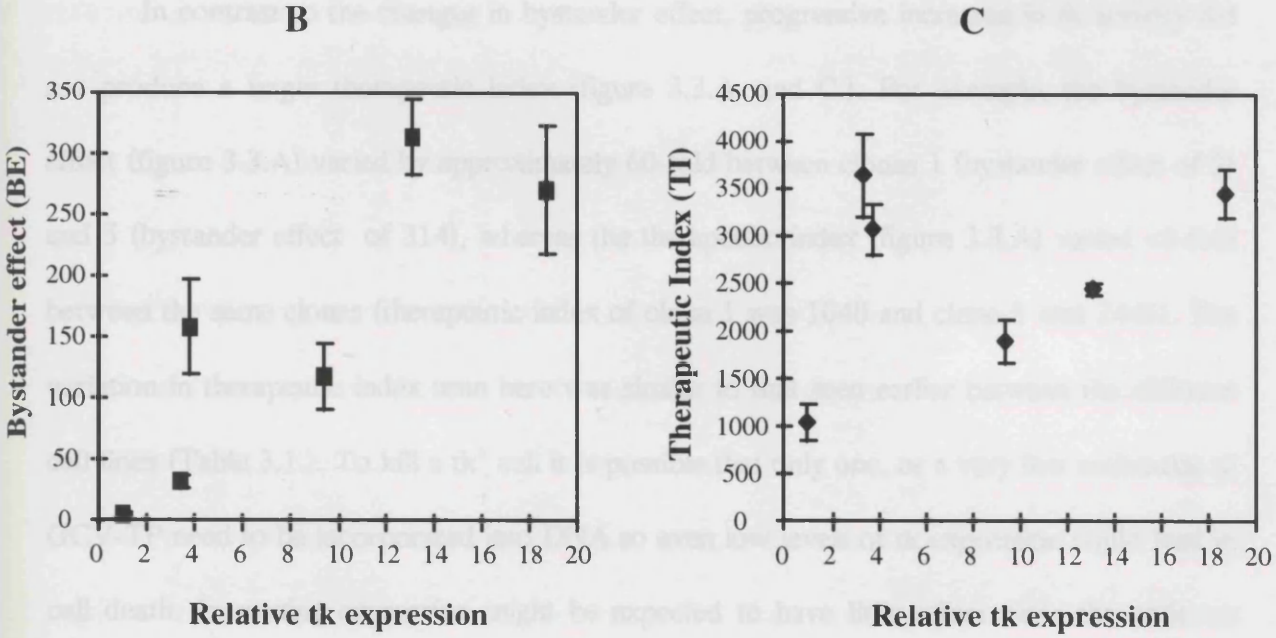


Figure 3.3. Effect of increasing tk expression on bystander effect and therapeutic index in BHK cells. BHK cells were transfected with the pGK-tk plasmid which carries neo and tk genes. Clones showing resistance to 800µg/ml G418 and sensitivity to 10µM GCV were further assayed for tk activity (section 2.4.3.). GJIC was determined by Lucifer Yellow dye injections (section 2.2.12.). Data are from 10 separate, 2 minute, injections and represent mean +/- SE. Doubling time of cells, after initial seeding at 5 x 10^4 / ml was measured (section 2.2.5.). Data are mean +/- SE of 2 experiments. (A) Survival of separate and mixed (10% tk^+) cultures of BHK tk^+ and BHK tk^- was analysed as described in section 2.2.7. By calculation from each survival curve, the IC50 for wild type cells, (0% tk^+) transfected cells (100% tk^+) and mixed cultures (10% tk^+) was determined. From these values (B) bystander effect ($^{IC50\ 0\%\ tk+}/_{IC50\ 10\%\ tk+}$) and (C) therapeutic index ($^{IC50\ 0\%\ tk+}/_{IC50\ 100\%\ tk+}$) were calculated and plotted versus tk activity (relative to tk clone with lowest expression, see section 3.3.2.). Each data point represents mean +/- SEM (bars) of two independent experiments, each performed in triplicate and relative tk activity represents the mean of two independent experiments, each performed in duplicate.

There was a strong correlation (0.855) between bystander effect and relative tk activity (figure 3.3.A. and B.). This did not appear to be influenced by the variations in mitotic index and GJIC that occur. For example, a faster cell cycle may be expected to enhance GCV toxicity, and hence the bystander effect, as toxicity from GCV-triphosphate arises from incorporation into nascent DNA strands and inhibition of αDNA polymerase during S phase. Increased gap junctional communication would, if anything, be expected to enhance the bystander effect. However, this was not the case since clone 2 had a shorter doubling time and was better coupled than clone 6 (figure 3.3.A.) but higher tk activity was associated with a stronger bystander effect.

In contrast to the changes in bystander effect, progressive increases in tk activity did not produce a larger therapeutic index (figure 3.3.A. and C.). For example, the bystander effect (figure 3.3.A) varied by approximately 60-fold between clones 1 (bystander effect of 5) and 5 (bystander effect of 314), whereas the therapeutic index (figure 3.3.A) varied <3-fold between the same clones (therapeutic index of clone 1 was 1040 and clone 5 was 2441). The variation in therapeutic index seen here was similar to that seen earlier between the different cell lines (Table 3.1.). To kill a tk^+ cell it is possible that only one, or a very few molecules of GCV-TP need to be incorporated into DNA so even low levels of tk expression could lead to cell death. Increasing expression might be expected to have little effect since the cells are already being effectively killed. The maximum therapeutic index is reached with low tk expression (figure 3.3.C.). However, in the bystander effect, under the conditions described here (10% tk^+ cells), one tk^+ cell must kill 4 tk^- cells to reduce survival of the culture by 50%. Sufficient toxic metabolites must be transferred to the tk^- cells in the culture and this will be achieved more effectively when levels of tk are high. Other factors may ultimately become limiting, such as uptake of GCV, and a plateau of maximal effect will be reached. It has also been suggested that low levels of toxic metabolites can be tolerated in some cell lines and

buffering of toxicity by competition with dGTP may reduce the bystander effect (Wygoda et al, 1997). This will be considered further in the discussion of this chapter (section 3.4.)

If this model is true then the bystander effect or the therapeutic index might be expected to increase initially and then plateau in response to increasing tk expression. While the data may be consistent with this expectation, there is too much variation to support any such conclusion. Variations are to be expected as the bystander effect and therapeutic index are calculated as ratios of IC50s ($^{0\%tk+}$ / $_{10\%tk+}$ and $^{0\%tk+}$ / $_{100\%tk+}$, respectively) and any differences due to biological variation, or experimental error, will be amplified.

From these data it is possible to conclude that low levels of tk activity result in little or no bystander effect, but increasing activity leads to stronger bystander killing.

3.3.3. Can toxicity be transferred via the medium?

Previous reports have demonstrated cell contact is required for a tk / GCV bystander effect with fibrosarcoma or Kirsten ras transformed cells in culture (Bi et al, 1993; Freeman et al, 1993). For this reason, the survival experiments described in section 3.3.1. were designed to optimise contact. The results in previous sections indicate that gap junctions may be required for the effect. If this is correct then toxicity would not be expected to be transferred via the medium. If it is not correct and toxicity is transferred via the medium, bystander killing would be expected in L929 or MCF-7 mixed cultures. There is some evidence in support of medium transfer as Kuriyama et al (1995) showed that medium from tk^+ cells treated with GCV could kill a proportion of the tk^- culture. They hypothesised that cyclic GCV-MP or a cytotoxic cytokine were involved (section 3.3.1.). If these toxic products are not produced by

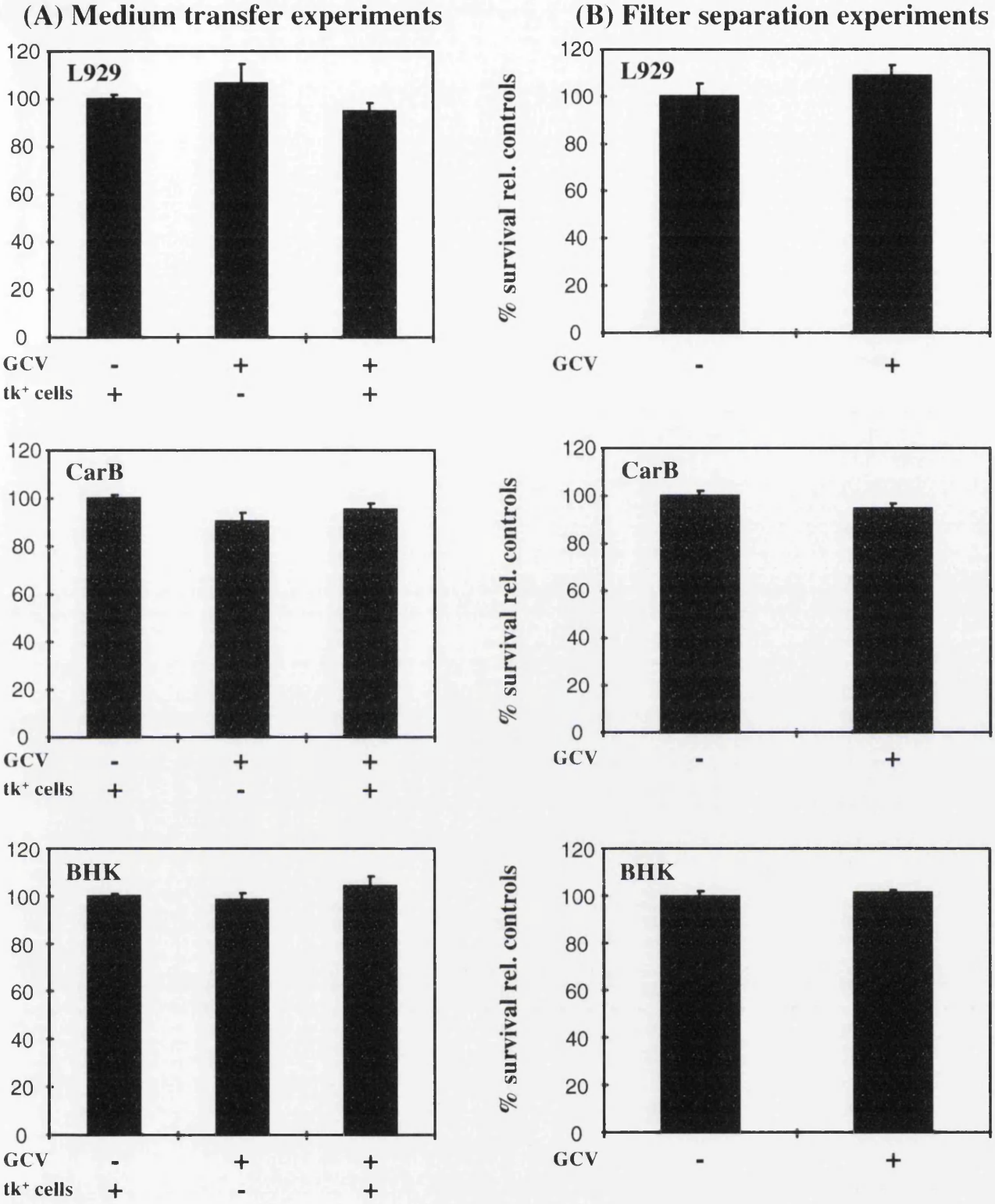


Figure 3.4. Transfer of toxicity in the tk / GCV bystander effect does not involve soluble metabolites. **(A)** tk^+ cells were seeded (L929 and CarB, 1 x 10^5; BHK, 5 x 10^4) cells / ml in 24 well plates and treated with 10µM GCV for 48 hr. The medium from drug treated cells was transferred to untransfected tk^- cells and incubated for 2 days. Survival was assayed using standard protocol (see section 2.2.7.) and compared to survival of controls. Treatments in each experiment are summarised below the graphs. **(B)** untransfected tk^- cells were seeded (L929, 5 x 10^4; CarB, 7 x 10^4; BHK, 2 x 10^4) in 0.5 ml in 24 well plates. 0.02µm filter inserts were placed in each well and an equal number of tk^+ cells added to the insert. 10µM GCV was added to the media and cells were incubated for 3 days. Survival was assayed using standard protocol (section 2.2.7.) and compared to untreated controls. Each data point represents mean +/- SEM (bars) of two independent experiments, each performed in triplicate.

L929 tk^+ and MCF-7 tk^+ cells, this may account for the lack of bystander effect in these cells. Therefore, transfer of toxicity via the medium was investigated.

In medium transfer experiments, cultures of tk^+ cells were treated with 10μM GCV for 2 days, a concentration known to produce a bystander effect (section 3.3.1.; figure 3.2). The medium was then transferred to tk^- cells which were incubated for 48 hr. Survival of tk^- cells was measured after transfer to 96 well plates, as described in section 2.2.7. Figure 3.4.A. shows there was no significant difference in survival, compared to controls treated with GCV, for the cell lines tested (L929, CarB and BHK).

The failure to transfer toxicity, via the medium, from CarB tk^+ and BHK tk^+ cell lines was not due to degradation of GCV or toxic metabolites. Medium containing 10μM GCV, which has been preincubated for 48 hr, was still toxic to tk^+ cultures.

Separation of tk^+ and tk^- cells, by an Anopore filter, also prevented a bystander effect (figure 3.4.B.). The filter was permeable to GCV because adding prodrug to the medium of tk^- cells, killed tk^+ cells, which were on the opposite side of the membrane. Any toxicity produced by the tk^+ cells should have passed rapidly to the tk^- cells so degradation of potentially toxic metabolites was less likely to occur. This experiment used equal numbers of tk^+ and tk^- cells but there was no detectable bystander effect. In contrast, when tk^+ and tk^- cells were in contact, and formed gap junctions, the same concentration of GCV (10μM) almost completely killed cultures containing only 20% tk^+ cells (figure 3.2.).

These experiments have shown that a detectable level of toxicity is not transferred via the medium and, in culture, cell contact is a requirement for the tk / GCV bystander effect. This leaves gap junctions or some form of vesicular transfer as the likely the mechanism of the bystander effect and since differences in apoptosis may account for the lack of vesicle production in some cell lines, this is explored below.

3.3.4. The role of apoptosis in the tk / GCV bystander effect

Apoptosis often occurs in response to toxic agents, for example, chemotherapeutic agents (Cece et al, 1995). Apoptosis also occurs when tk^+ cells are treated with GCV and this has led to the suggestion that phagocytosis of apoptotic vesicles is a mechanism of transfer of toxicity (Freeman et al, 1993). If L929 tk^+ and MCF-7 tk^+ cells do not undergo apoptosis and produce vesicles in the same way as the other cell lines examined, this could account for a lack of bystander effect.

In the report by Freeman et al (1993) cell contact was shown to be required for a bystander effect. Since contact is necessary for transfer via gap junctions, and may be required for transfer by vesicles, this raises the problem of how to differentiate between these mechanisms. Fortunately, shedding of vesicles occurs late during apoptosis, whereas other events in programmed cell death, such as DNA cleavage, occur much earlier. For example, in bovine epithelial cells treated with ricin, changes in DNA have been shown to occur at 7 hr while release of vesicles was seen at times after 16 hr (Hughes et al, 1996; section 1.11.2.). Determining the time that DNA damage occurs should define the earliest time of vesicle release. Transfer via gap junctions should, on the other hand, occur much faster. For example, extensive nucleotide transfer between coupled cells occurs in one hr (Sheridan et al, 1979) and in section 3.2.2. Lucifer Yellow spreads to >20 cells in 2 minutes. Determining the time of transfer of toxicity should distinguish which mechanism is involved. Therefore, experiments were designed to answer the following questions:-

1) Do all the tk^+ cell lines die by apoptosis after GCV treatment? If only cells that show a bystander effect die this way, this may account for the lack of bystander killing in L929 and MCF-7 cultures.

2) When does apoptosis occur? If apoptosis occurs at a much later time in cells that do not show a bystander effect this could account for differences in bystander killing.

3) When does transfer of toxicity occur? Early transfer prior to the onset of apoptosis would favour GJIC as the basis of the bystander effect.

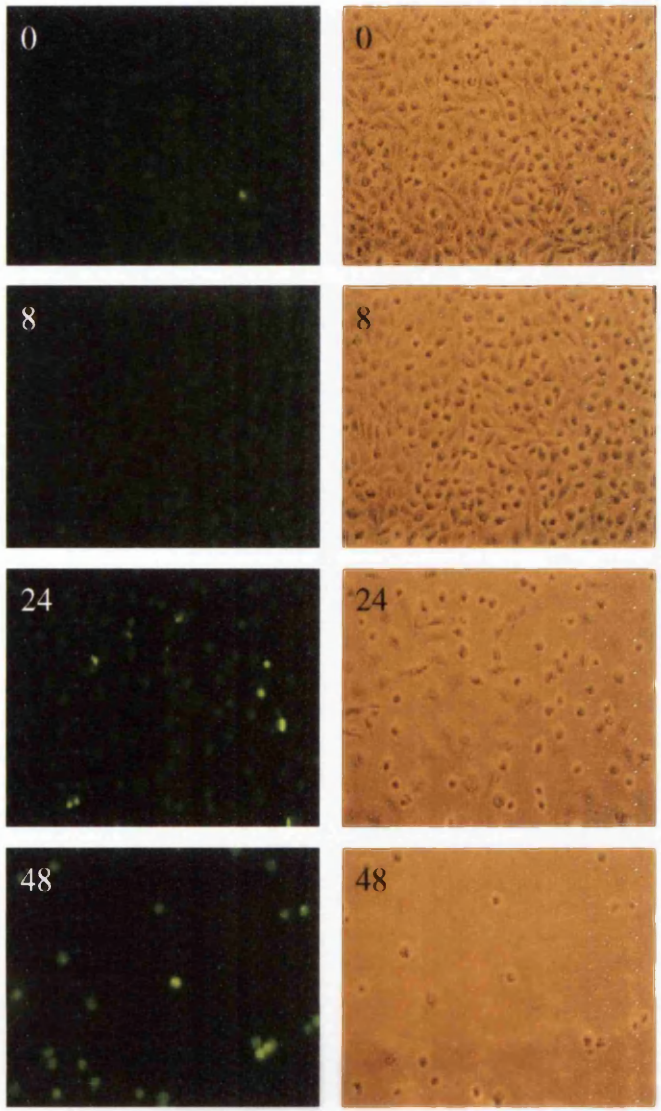
Some of the experiments below rely on colony formation. L929, CarB and BHK cells readily form colonies and these cells do (CarB and BHK) or do not (L929) show a bystander effect. Therefore these cell lines have been selected as representatives to study the tk / GCV bystander effect further. (MCF-7 cells form colonies more slowly, while B9 cells can clump, making studies such as flow cytometry difficult, see below).

The time course of cell death of tk^+ cells in response to GCV was determined using a terminal transferase assay. Free ends of DNA produced by nuclease activity during apoptosis and necrosis incorporate fluorescein labelled nucleotides, which allows cells with fragmented DNA to be seen under appropriate UV illumination. Cleavage of DNA occurs in apoptosis and necrosis (see below) therefore this method detects both events. While GCV treatment had no detectable effect on tk^- cells at any of the time points examined, tk^+ cells showed DNA damage at times after 8 hr and morphological evidence of cellular damage was first evident at 16 to 24 hr (figures 3.5.a.b. and c. part A). The time course of cellular degeneration in L929 tk^+ and CarB tk^+ cells was similar but the events occurred earlier in BHK tk^+ cells. In each cell line a marked reduction in cell number occurred with time. Closer examination of the photomicrographs in figures 3.5 (a, b and c. part A) reveals that cell shrinkage (see phase contrast photomicrograph) and condensation of DNA (see immunofluorescence photomicrograph), have occurred, which are indicative of apoptosis. However, some cells appear swollen and enlarged, which suggests necrosis (see section 1.11.2.).

Apoptosis is associated with the appearance of ≈180 bp DNA fragments which represent cleavage of linker regions between nucleosomes by a specific endonuclease. This

Figure 3.5a. A population of L929 tk^+ cells treated with GCV die by apoptosis. **(A)** In situ analysis of GCV induced cell death. L929 tk^+ cells were seeded at 1 x 10^5 cells / ml in 2 well chamber slides. 24 hr later, cells were treated with 20μM GCV for 0, 8, 24 or 48 hr. Cells were stained using an Apotag detection kit as described in section 2.6.1. Immunofluorescence and phase contrast photomicrographs are shown. Untreated cultures show only background staining and positive staining is seen after 8 hr. This demonstrates that apoptosis or necrosis occurred. **(B)** Presence of DNA fragmentation was determined by agarose gel electrophoresis of ^{32}P end labelled samples. Cultures of L929 or L929 tk^+ cells were incubated with 20μM GCV for the times indicated above. Genomic DNA was isolated from these cells and free ends of DNA were labelled with $\alpha[^{32}P]$-dCTP as described in section 2.6.2. **b(i)** UV illumination of ethidium bromide stained DNA shows equal loading between samples. **b(ii)** The gel was dried and exposed to autoradiographic film. L929 parental cells (tk^-) and untreated tk^+ cells show no evidence of specific DNA fragmentation, whereas cells treated for 24 and 48 hr do (arrows). This demonstrates that some cell death occurs by apoptosis.

(A) Detection of DNA strand breakage in L929 tk⁺ cells using Apotag staining



(B) Detection of DNA laddering in L929 tk⁺ cells

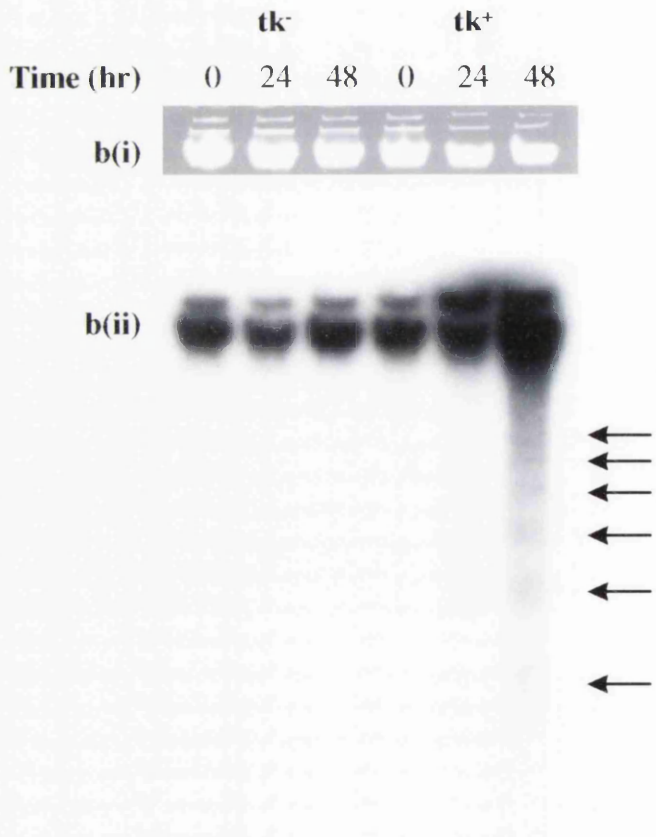


Figure 3.5a. Determination of the time course of apoptosis in L929 tk⁺ cells

Figure 3.5b. A population of CarB tk^+ cells treated with GCV die by apoptosis. **(A)** In situ analysis of GCV induced cell death. CarB tk^+ cells were seeded at 1.5 x 10^5 cells / ml in 2 well chamber slides. 24 hr later, cells were treated with 20μM GCV for 0, 8, 24 or 48 hr. Cells were stained using an Apotag detection kit as described in section 2.6.1. Immunofluorescence and phase contrast photomicrographs are shown. Untreated cultures show only background staining and positive staining is seen after 8 hr. This demonstrates that apoptosis or necrosis occurred. **(B)** Presence of DNA fragmentation was determined by agarose gel electrophoresis of ^{32}P end labelled samples. Cultures of CarB or CarB tk^+ cells were incubated with 20μM GCV for the times indicated above. Genomic DNA was isolated from these cells and free ends of DNA were labelled with $\alpha[^{32}P]$-dCTP as described in section 2.6.2. **b(i)** UV illumination of the ethidium bromide stained DNA shows equal loading between samples. **b(ii)** The gel was dried and exposed to autoradiographic film. CarB parental cells (tk^-) and untreated tk^+ cells show no evidence of specific DNA fragmentation, whereas cells treated for 24 and 48 hr do (arrows). This demonstrates that some cell death occurs by apoptosis.

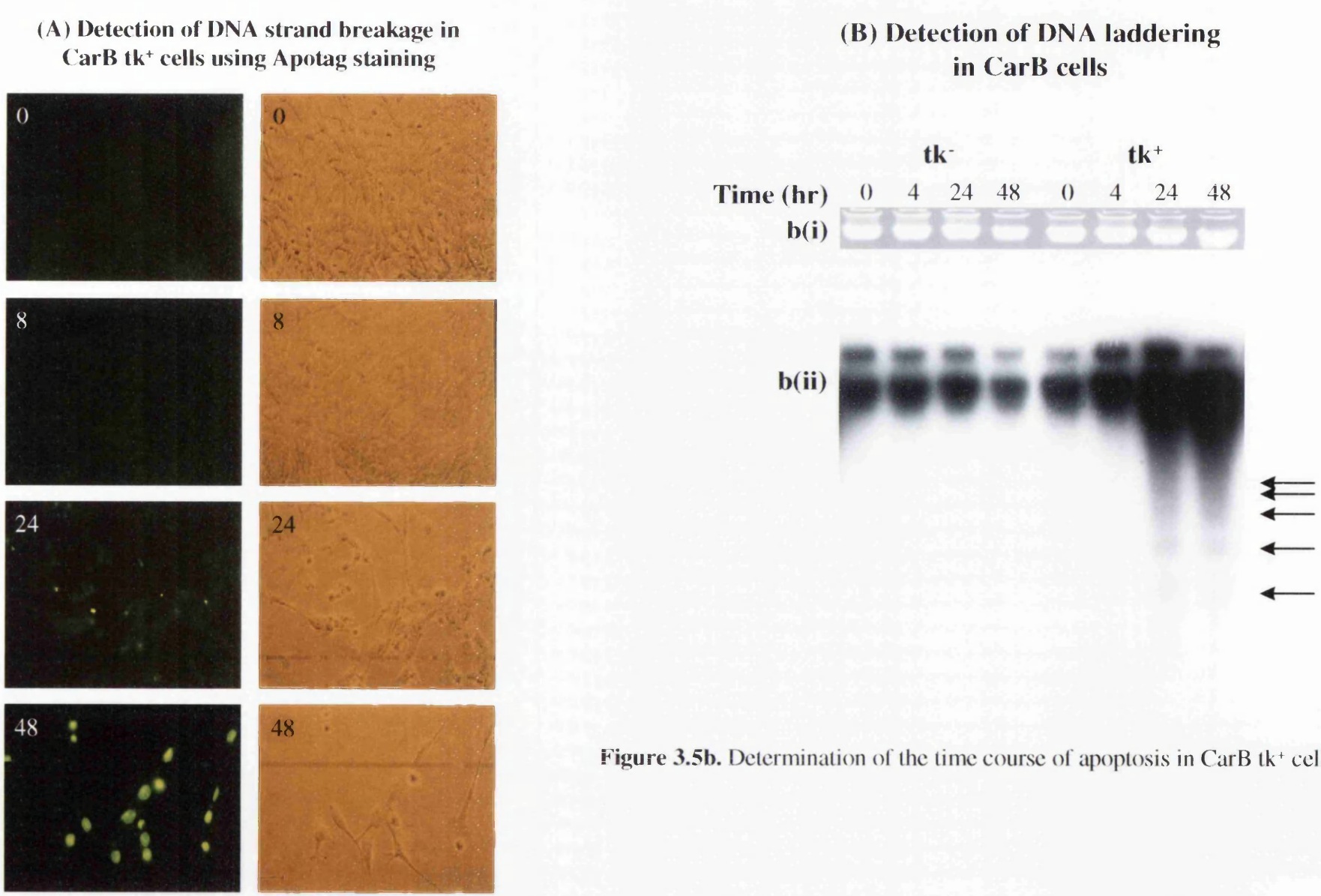


Figure 3.5b. Determination of the time course of apoptosis in CarB tk+ cells.

Figure 3.5c. A population of BHK tk^+ cells treated with GCV die by apoptosis. **(A)** In situ analysis of GCV induced cell death. BHK tk^+ cells were seeded at 5 x 10^4 cells / ml in 2 well chamber slides. 24 hr later, cells were treated with 20μM GCV for 0, 4, 8 or 24 hr. Cells were stained using an Apotag detection kit as described in section 2.6.1. Immunofluorescence and phase contrast photomicrographs are shown. Untreated cultures show only background staining and positive staining is seen after 8 hr. This demonstrates that apoptosis or necrosis occurred. **(B)** Presence of DNA fragmentation was determined by agarose gel electrophoresis of ^{32}P end labelled samples. Cultures of BHK or BHK tk^+ cells were incubated with 20μM GCV for the times indicated above. Genomic DNA was isolated from these cells and free ends of DNA were labelled with $\alpha[^{32}P]$-dCTP as described in section 2.6.2. **b(i)** UV illumination of the ethidium bromide stained DNA shows equal loading between samples. **b(ii)** The gel was dried and exposed to autoradiographic film. BHK parental cells (tk^-) and untreated tk^+ cells show no evidence of specific DNA fragmentation, whereas cells treated for 8 and 24 hr do (arrows). This demonstrates that some cell death occurs by apoptosis.

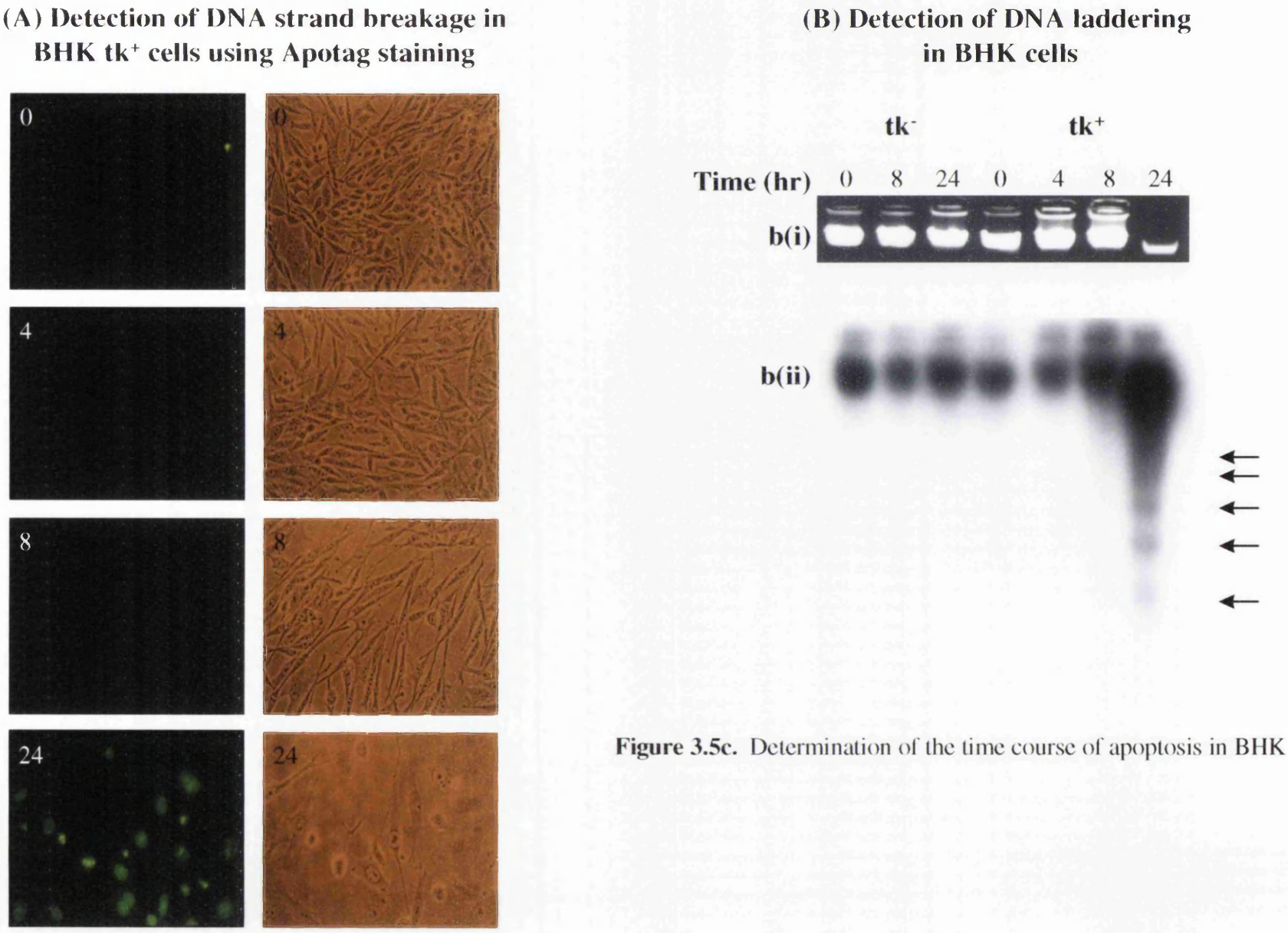


Figure 3.5c. Determination of the time course of apoptosis in BHK tk+ cells.

contrasts to necrosis where non specific nucleases digest DNA generating a range of fragment sizes. Separation of DNA fragments by agarose gel electrophoresis, reveals laddering or smearing which indicate apoptosis or necrosis, respectively.

To determine which event(s) was predominant, genomic DNA was isolated from tk^+ or tk^- cells (L929, CarB and BHK) that had been treated with GCV for different times up to 48 hr (figure 3.5. a, b. and c. part B). Free ends of DNA were labelled with $\alpha[^{32}P]$-dCTP and separated by electrophoresis (section 2.6.2.). Radiolabelling enhances the sensitivity of this assay. No change in DNA integrity was visible in the ethidium bromide stained gel, but did show equal loading between lanes (figures 3.5. a, b and c. part Bi). Exposure to autoradiographic film revealed laddering at times after 4 hr of GCV treatment in tk^+ cells (figures 3.5. a, b and c. part Bii). Apoptosis occurred in each of the cell lines but appeared earliest in CarB tk^+ and BHK tk^+ cells, and latest in L929 tk^+ cells. All the samples that showed laddering also showed smearing, which suggests that necrosis occurred in parallel.

Flow cytometric analysis of cells stained with propidium iodide (PI) allows changes in DNA content to be studied. When intercalated with double stranded DNA, PI fluoresces with exposure to 488nm excitation. The level of fluorescence is proportional to DNA content of individual cells. In addition, cell size increases during the cell cycle to a maximum prior to mitosis. Plotting fluorescence against cell size indicates the proportion of cells in each phase. Any change in the distribution of cells indicates a change in DNA content.

In earlier experiments, which used labelling of fragmented DNA to assess the time course of apoptosis, degeneration occurred more rapidly in BHK tk^+ cells, therefore an additional time point was included for flow cytometric analysis of this cell line. The results in figure 3.6 show changes in DNA content (as changes in the flow cytometric profile) were detectable at times after 2 hr of GCV treatment of BHK tk^+ cells or at times after 4 hr treatment of L929 tk^+ and CarB tk^+ cells. These data are consistent with those in figure 3.5

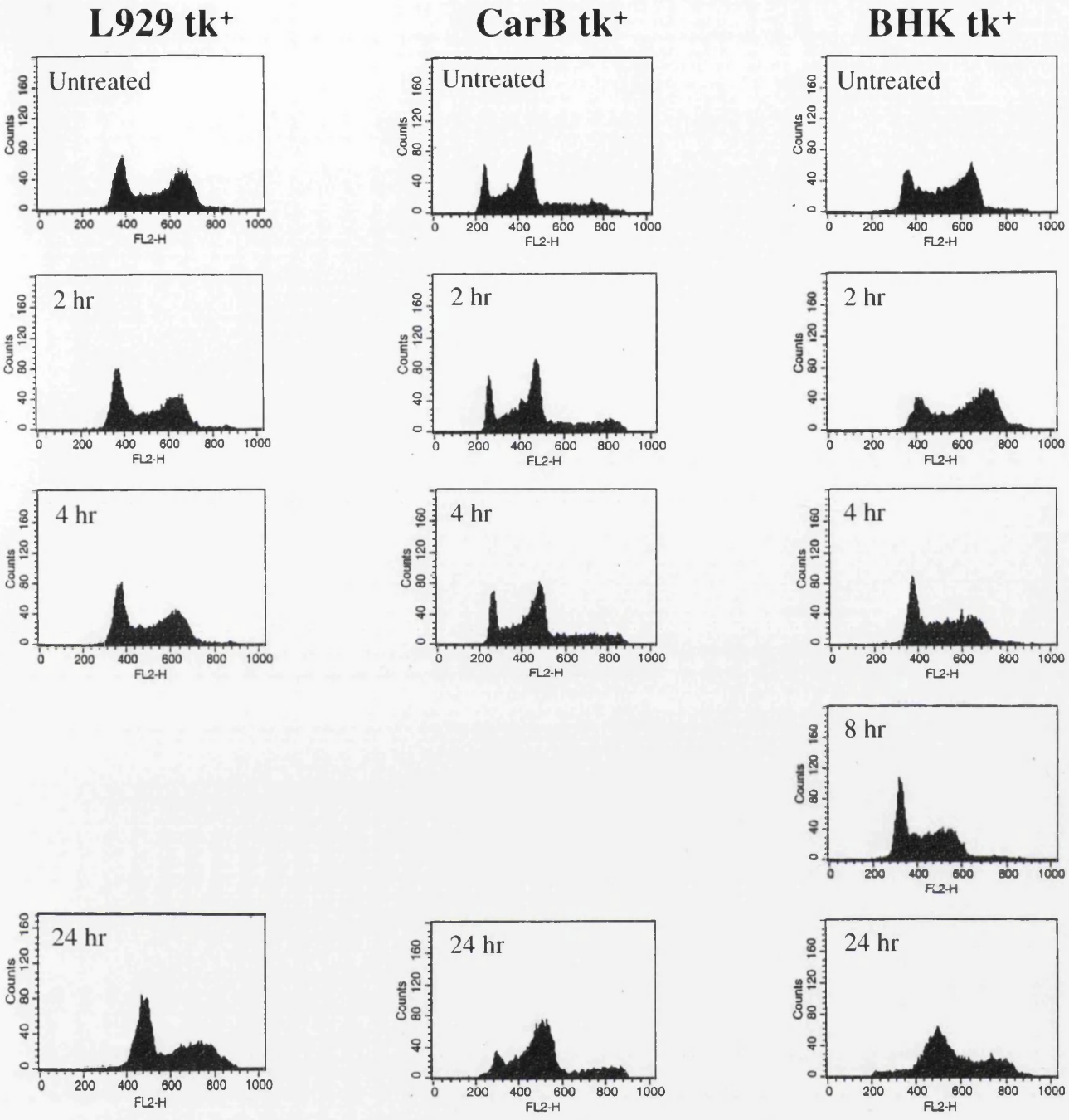


Figure 3.6. The earliest changes in DNA content of GCV treated tk^+ cells occurs at times after 2 hr. tk^+ cells were seeded (L929 tk^+ and CarB tk^+, 1 x 10^5; BHK tk^+ 5 x 10^4 cells / ml) in 90mm tissue culture plates and allowed to settle for 24 hr. Cultures were treated with 20μM GCV for times indicated above, (the additional time point for BHK tk^+ cells was due to the shorter division time of this cell line, see figure 3.1b.). Cells were harvested and prepared for flow cytometry as described in section 2.6.3. DNA content of untreated and treated cells was quantified by collecting the emissions from propidium iodide stained samples that had been excited by 488 nm light. The data from each figure have been collected from 10,000 cells / sample and are representative of three independent experiments.

and suggest the shorter doubling time (more S-phase entry) of BHK cells leads to a faster activation of cellular degeneration.

An apoptotic peak, which has been reported by others in response to toxic agents (Cece et al, 1995), was not seen in the histograms from GCV treated tk^+ cells here. Previous studies have shown that some cells dying by apoptosis release DNA containing vesicles which, due to their small size, generate a peak to the left of the histogram (the larger the particle, the further to the right the signal). The absence of such an apoptotic peak in the histograms of the GCV treated tk^+ cells, and the detection of laddering only by a sensitive radiolabelling assay, suggest that only a small proportion of the cells are undergoing apoptosis at the time points investigated.

Collectively these data demonstrate the mechanism of cell death in cells that do and do not show a bystander effect occurs, in part, by apoptosis. The time courses of these events are similar for L929 tk^+ and CarB tk^+ cells, and there are no obvious differences that can be related to the differences in bystander effect. Changes in BHK tk^+ cells occur earlier. However, no changes in morphology or DNA content of any of the cells examined can be detected at 2 or 4 hr and it seems reasonable to conclude that apoptotic vesicles will not be released before these times. If transfer of toxicity occurs at these early times, it would provide evidence that apoptotic vesicles are not involved.

To examine the time at which toxicity is transferred, the effect of GCV treatment on colony formation in separate cultures, was investigated, for different times up to 4 hr,. Treatment of tk^+ cells with GCV for 2 hr resulted in >90% to cell death and complete killing was seen after 4 hr treatment (figure 3.7a.). During these periods of treatment, only a fraction of the cells can be expected to be in S-phase, which is when toxicity from GCV-TP occurs. The remaining cells, that were not synthesising DNA during treatment, can only be killed if, at a later time they enter S-phase in the presence of toxic metabolites and this could occur either by

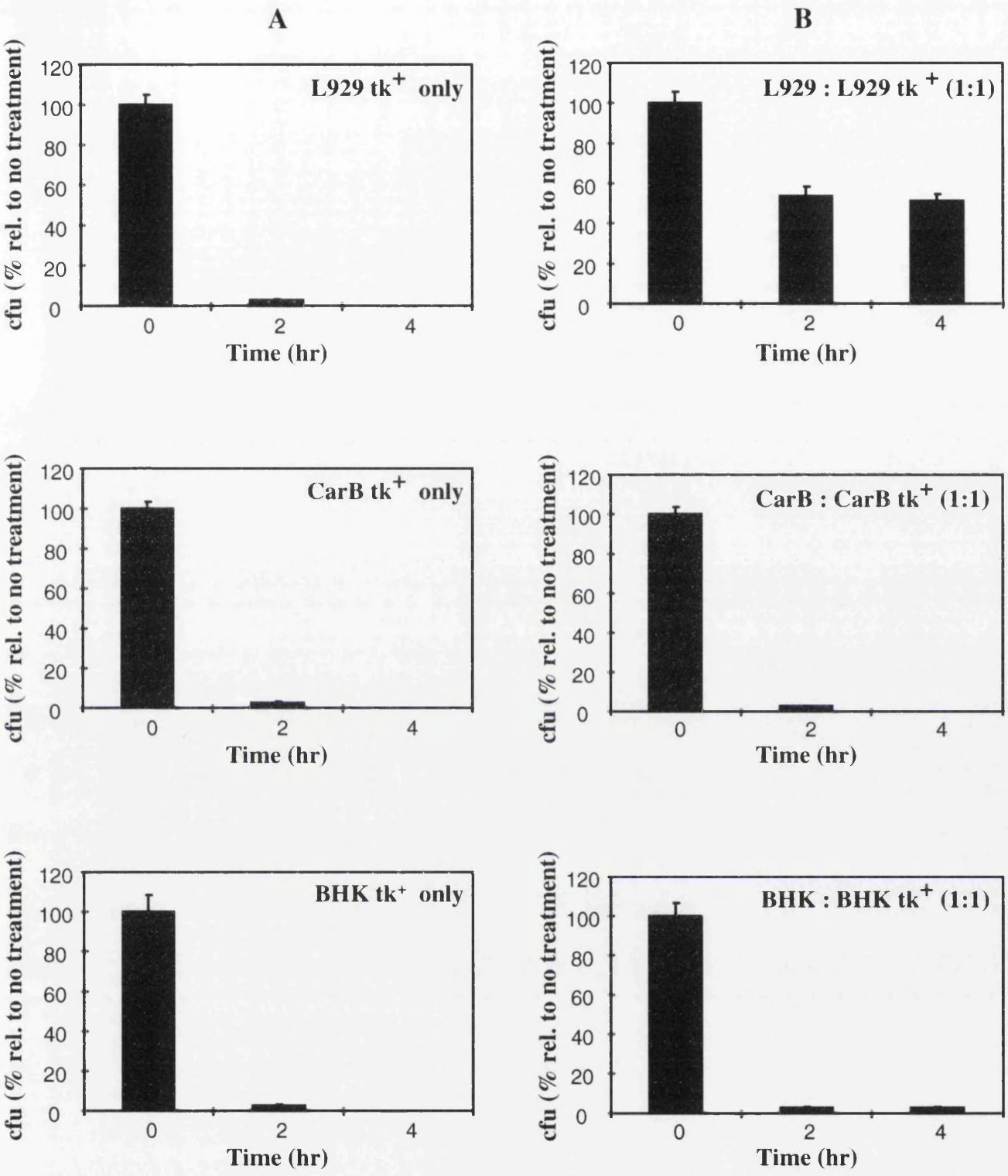


Figure 3.7. Toxicity in the tk / GCV bystander effect is transferred within 2 hr of applying GCV. tk^+ cells alone **(A)** or tk^+ and tk^- mixed (1:1) cultures **(B)** were seeded (L929, 1 x 10^6; CarB, 2 x 10^6; BHK, 5 x 10^5 cells / well) in 35 mm plates and allowed to settle for 4 hr. Cells were untreated (0 hr) or treated for 2 or 4 hr with 20μM GCV. Cells were released with trypsin and aliquots of the cell suspension were transferred to 90 mm plates containing full medium (no GCV) in order to test the ability of cells to form colonies. When visible colonies had formed they were stained with Giemsa. Colonies were counted and survival calculated as a percentage relative to untreated cultures. Data represent the mean +/- SEM of three independent experiments. In the cells lines tested >95% of tk^+ cells are killed by 2 hr of GCV, whereas in mixed cultures tk^- cells are killed only when cells communicate (CarB and BHK, not L929 cells).

the persistence of GCV-TP or by further production of GCV-TP from residual GCV. In the protocol described above, GCV treated tk^+ cells were transferred to medium without prodrug, and cellular GCV might be expected to become diluted by equilibrium with the surrounding medium. Therefore, it is more likely that persistence of GCV-phosphates, which cannot cross that cell membrane, were the cause the death, and this suggests that these metabolites may have a relatively long half life.

To determine whether transfer of toxicity can occur during 2 or 4 hr of treatment, mixed cultures (1:1) of cells that do not (L929 and L929 tk^+) or do (CarB and CarB tk^+ or BHK and BHK tk^+) show a bystander effect were seeded to give subconfluent cultures after 4 hr. Cultures were treated with 20μM GCV for 2 or 4 hr, trypsinised and colony formation measured (figure 3.7a.). At both times GCV killed only approximately half the L929 cells (the tk^+) but most of the CarB and BHK cells, compared to untreated controls. The phenotype of the surviving L929 cells was shown to be tk^- since in parallel experiments, treatment of mixed cultures of L929 $tk^-\beta gal^+$ and L929 $tk^+\beta gal^-$ cells resulted only in survival of cells that stained positive with x-gal (section 2.2.13.).

It is also possible that the bystander effect seen in the CarB and BHK cells depends on some form of cytoplasmic budding and uptake that occurs in normal cultures. Like any form of vesicular transfer, such a mechanism could result in tk^- cells gaining tk activity. To examine this possibility (transfer of tk enzyme or genetic information to make the enzyme), tk^+ and tk^- (L929, CarB or BHK) mixed cultures, at the same densities as before, were left for 8 hr, trypsinised, plated at low density (to prevent contact) and then treated with 20μM GCV. Only half the cells (compared to untreated controls) were killed in each mixed culture (figure 3.7b.). These data show that after coculture, under conditions that generate a pronounced bystander effect, negligible tk activity was transferred from the tk^+ to the tk^- cells.

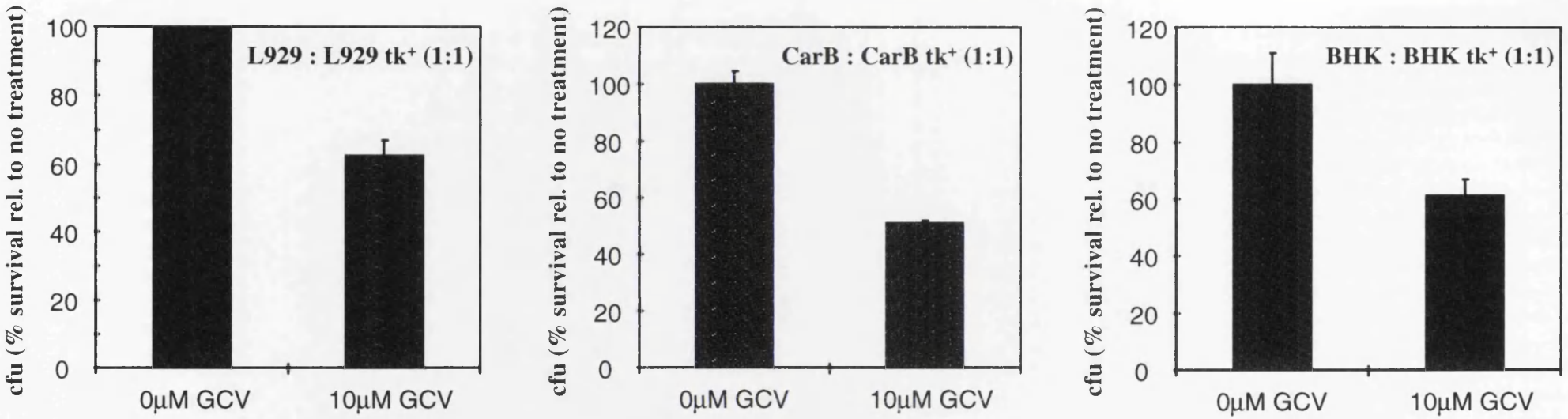


Figure 3.7b. Toxicity in the tk / GCV bystander effect is not transferred by vesicles. Mixed cultures (1:1) of tk^+ and tk^- cells were seeded (L929, 1 x 10^6; CarB, 2 x 10^6; BHK, 5 x 10^5 cells / well) in 35 mm plates and allowed to settle and grow for 8 hr. Cells were released with trypsin and aliquots of the cell suspension were transferred to 90 mm plates containing 10μM GCV in order to test the ability to form colonies. When visible colonies had formed they were stained with Giemsa. Colonies were counted and survival calculated as a percentage relative to untreated cultures. Data represent the mean +/- SEM of two independent experiments. In the cell lines tested 50-60% cell death occurred, showing that vesicles do not transfer tk mRNA or protein to untransfected cells.

Taken together, the above data show that transfer of toxicity in the tk / GCV system is not via the medium and is unlikely to involve uptake of apoptotic or other vesicles. Apoptosis did occur in all tk^+ cell lines treated with GCV, but mixed cultures of tk^+ and tk^- cells were committed to death before apoptosis could be detected. The most likely explanation of the bystander effect in this system is transfer of toxic metabolites via gap junctions.

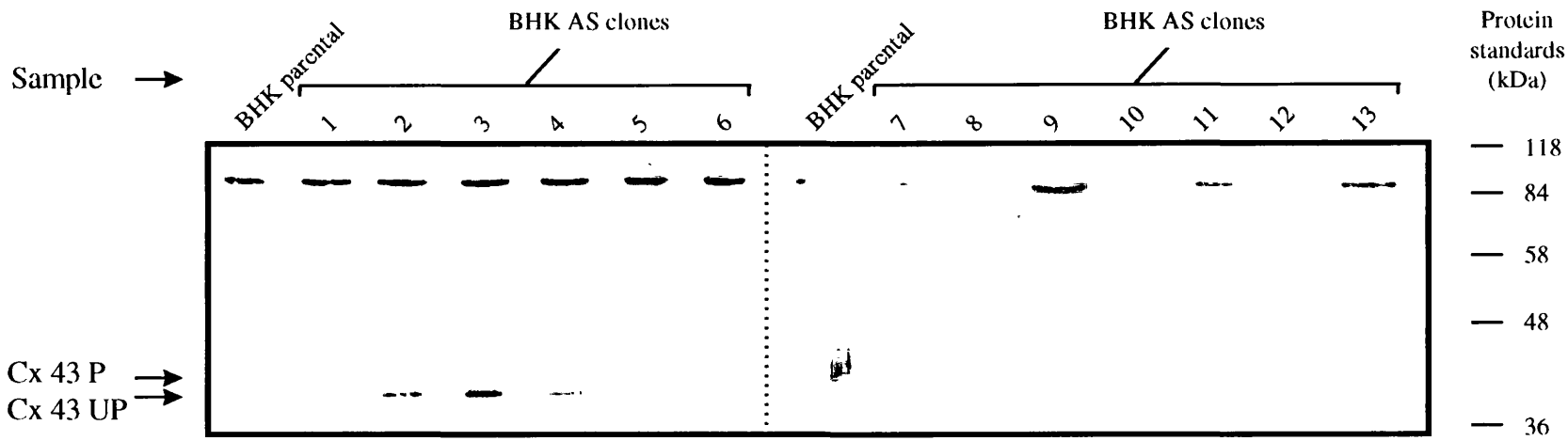
3.3.5. Modulation of GJIC and the tk / GCV bystander effect

The correlation between the tk / GCV bystander effect and GJIC would be enhanced if it were possible to selectively modulate communication with genetic manipulation or specific inhibitors. Expression of genes that enhance gap junction formation in poorly coupled cells can markedly increase the level of junctional communication. For example, expression of E-cadherin increased coupling of CarB cells up to >5-fold (figure 3.1.) while in Hela cells, expression of connexin 43 (Cx43) resulted in a 20- to 40-fold increase (Mesnil et al, 1996). In L929 and MCF-7 cells, which do not express E-cadherin and Cx43, respectively, there is no detectable coupling (figure 3.1.). Expression of the appropriate functional genes in these cells, however, fails to induce communication and it appears that the defects are more complex (Pitts, unpublished data). Some other factor(s), not yet identified, is required for proper formation of gap junctions in these cell types.

Decreasing the level of communication may provide an alternative way to study the correlation. However, currently available chemical modulators such as forskolin are not specific to gap junctions and also inhibit phagocytosis (Nambu et al, 1989) while others, such as TPA (12-O-tetradecanyol phorbol-13-acetate), which are also not specific, fail to completely inhibit coupling in many cell lines (Rivedal and Sanner, 1992). Another approach is the use of antisense constructs. This approach has proved effective, and specific, in reducing

levels of several proteins, for example, the proto-oncogene bcl-2 (see section 1.6.). Goldberg et al (1994) demonstrated a full length Cx43 antisense (Cx43-AS) construct can reduce junctional communication in Rat 1 cells in culture. Lucifer Yellow dye spread was reduced from 49 cells in parental cultures to 1 cell in an antisense expressing clone, a reduction of 98%. If communication of the cells in the present study could be reduced this effectively, coupling would be close to zero. This would allow further examination of the role of GJIC in the bystander effect. However, connexins are a family of proteins and it is possible there will be redundancy in function. It may be important to select a cell line with a limited number of connexins expressed. BHK cells express Cx43, but many connexins, such as Cx-26, -31, -31.1, -32, -37, -40, -46 and -50, are not expressed (Davis et al, 1995). As the sequences of hamster (BHK cells) and rat (Rat-1 cells) Cx43 show 95% homology, and the Cx43 AS plasmid used by Goldberg (above) was successful in Rat-1 cells, the same construct was transfected into BHK cells. Cells with this plasmid can be selected for with G418. Resistant clones were picked and complete lysates were resolved by SDS-PAGE. Western blot analysis revealed reduced levels of Cx43 protein in some of the clones (figure 3.8.A.). In parental cells, Cx43 is present in unphosphorylated and phosphorylated forms which migrate as separate bands with apparent molecular weights of 40-45 kDa, which is consistent with previous reports (Musil and Goodenough, 1991). Parental cells and most clones also express a protein of higher molecular weight (90 kDa) that reacts with the antibody. The appearance of a similar band has been reported previously (Davis et al, 1995). Since there is no correlation with level of Cx43, the presence of this band is likely to represent non specific binding. For example, clones 1 to 6 show similar levels of the 90kDa protein but Cx43 varies (figure 3.8.A). The level of coupling of 4 clones that showed marked decreases in Cx43 expression (3 have no detectable expression) was analysed by microinjection of Lucifer Yellow. However there was no reduction in coupling in these clones (Figure 3.8.B.). Decreasing Cx43 expression did not

A



B

Clone	BHK control	BHK Cx43 AS cl. 6	BHK Cx 43 AS cl. 7	BHK Cx 43 AS cl. 8	BHK Cx 43 AS cl. 9
GJIC	9.1 +/- 0.7	8.4 +/- 0.9	8.3 +/- 0.7	8.5 +/- 0.9	8.0 +/- 0.8

Figure 3.8. Down regulation of Connexin 43 in BHK cells does not significantly reduce GJIC. **(A)** Total cell lysates were prepared from parental cells or clones stably transfected with a connexin 43 antisense (Cx43 AS) construct. 40µg of total protein was resolved by SDS-PAGE and proteins transferred to nitrocellulose membrane by semi-dry electroblotting. Primary antibody (to Cx43) and secondary antibody were applied to the membrane as described in section 2.4.2. Position of antibody binding was determined using ECL detection and position of protein bands compared to protein standards which are shown to the right of the figure. Cx43 appears in two forms, unphosphorylated (Cx43 UP) and phosphorylated (Cx43 P). Parental cells and most clones expressed an unknown protein of approx. 90kDa. The dotted line indicates that samples were loaded on two separate gels in this figure they have been joined. **(B)** Clones which expressed reduced levels of Cx43 protein were analysed for presence of GJIC by 2 minute Lucifer Yellow dye injections, as described in section 2.2.12. Values represent minimum of 20 separate injections +/- SE. Dye spread was not significantly reduced when compared to parental cells.

reduce communication in BHK cells, and similar results were found with CarB and B9 cells (data not shown). The tk / GCV bystander effect cannot be investigated, in these cells, in this way.

3.3.6. Absence of a bystander effect in mixed cultures of cells that can form gap junctions and cells that cannot

The experiments in sections 3.3.1. to 3.3.4. provide evidence for a correlation between GJIC and the bystander effect in this system. However further support for gap junctions may be obtained by determining whether transfer of toxicity occurs in mixed cultures of cells that do or do not show a bystander effect. It has previously been demonstrated that coupling only occurs between different cell lines when both can form functional gap junctions, that is to say the inability to form gap junctions is dominant in mixed cultures (Pitts, 1971; Gilula et al, 1972). The role of gap junctions in the bystander effect would be supported if transfer of toxicity is not seen in cultures where only one cell line is coupled. Transfer of toxicity by vesicles or the medium should be non specific. Therefore CarB and L929 cells were selected to address this issue. CarB tk^+, but not L929 tk^+ cells, show a strong bystander effect when cultured with parental cells (Table 3.1.). In addition, these cells have similar population doubling times (figure 3.1.).

Mixed cultures (1:1) of L929 and CarB tk^+, CarB and L929 tk^+, CarB and CarB tk^+ or L929 and L929 tk^+ cells were seeded to give subconfluent cultures after 4 hr. Cultures were treated with 20μM GCV for 4 hr, trypsinised and colony formation examined (figure 3.9.). Colony formation, not an MTT assay, was used to assess survival of cultures as CarB and L929 cells metabolise MTT at different rates so comparing treated and untreated mixed cultures would give false levels of survival. The cultures of CarB and CarB tk^+ (>95% killing)

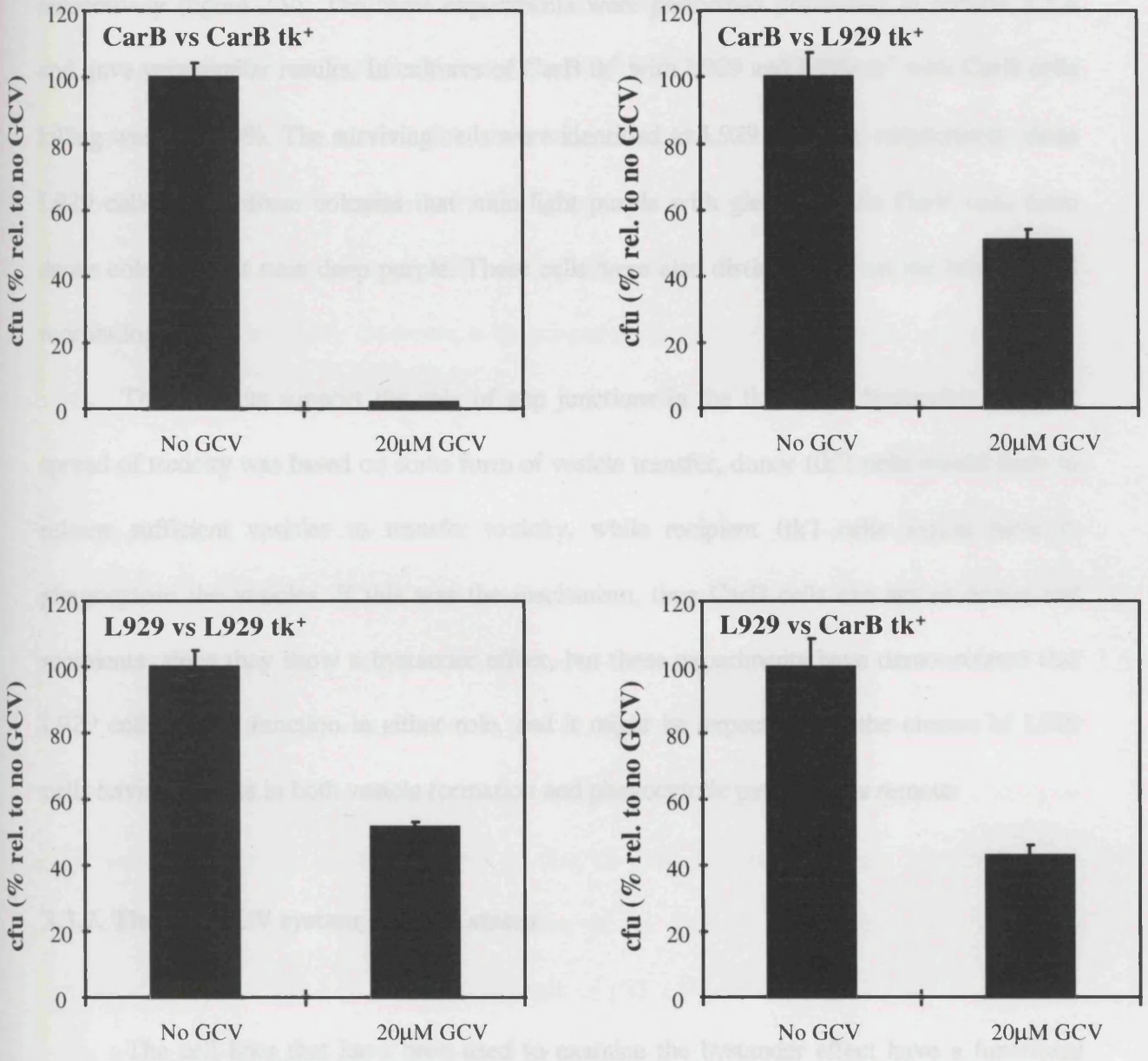


Figure 3.9. GJIC is required in tk^+ and tk^- cell populations for the tk / GCV bystander effect. Mixed cultures (1:1) of tk^+ and tk^- cells indicated in each of the figures above were seeded (1 x 10^6 cells / well) in 35 mm plates. 24 hr later cells were treated with 20μM GCV for 4 hr. After this time cells were released with trypsin and aliquots of the cell suspension were transferred to 90mm plates containing full medium (no GCV) in order to test the ability of cells to form colonies. When visible colonies had formed they were stained with Giemsa. Colonies were counted and survival calculated as a percentage of untreated cultures. Data represent mean +/- SE of one experiment performed in triplicate. CarB cells have GJIC and L929 cells do not. A bystander effect is only seen in CarB with CarB tk cells.

or L929 and L929 tk^+ (≈50% killing) were controls with or without bystander effects, respectively (figure 3.9). The same experiments were performed previously in section 3.3.4 and gave very similar results. In cultures of CarB tk^+ with L929 and L929 tk^+ with CarB cells killing was ≈50-60%. The surviving cells were identified as L929 or CarB, respectively, since L929 cells form diffuse colonies that stain light purple with giemsa, while CarB cells form dense colonies that stain deep purple. These cells were also distinguished on the basis of cell morphology.

These results support the role of gap junctions in the tk / GCV bystander effect. If spread of toxicity was based on some form of vesicle transfer, donor (tk^+) cells would have to release sufficient vesicles to transfer toxicity, while recipient (tk^-) cells would have to phagocytose the vesicles. If this was the mechanism, then CarB cells can act as donor and recipients, since they show a bystander effect, but these experiments have demonstrated that L929 cells cannot function in either role, and it might be expected that the chance of L929 cells having defects in both vesicle formation and phagocytotic pathways, is remote.

3.3.7. The tk / GCV system and p53 status

The cell lines that have been used to examine the bystander effect have a functional p53 gene (Blagosklonny et al, 1995; Schulze-Lutum et al, 1994; Lee et al, 1996). This protein is important in the regulation of the cell cycle in response to DNA damage and may be required for apoptosis in some cell types (Kastan et al, 1991). It is possible that GCV-triphosphate activates cell death pathways which require p53. The anticipated use of the tk / GCV system is for cancer treatment, but many cancers, for example >50% of non small cell lung carcinomas, lack p53 (Ngugen et al, 1997) and it is important to determine whether tk^+ cells without functional p53 are killed by GCV.

B95 fibroblasts are derived from a p53 knockout mouse (Alan Balmain, CRC Beatson). The p53 gene has been disrupted by insertion of a neo^R marker. The neo gene is expressed and the B95 $p53^-$ cells are resistant to G418. Therefore these cells were transfected with a plasmid carrying tk and $hygromycin^R$ genes. Clones resistant to 50μg / ml of hygromycin B were picked and screened for sensitivity to 5μM GCV. A clone with a similar growth rate to that of the parental cells was expanded. Survival of separate cultures of tk^+ and tk^- cells was measured after treatment with increasing concentrations of GCV, as described in section 2.2.7. (figure 3.10.A). Wild type cells were unaffected by 10μM GCV, while tk^+ cells were completely inhibited. Calculating the IC50 from each survival curve showed a therapeutic index of approximately 500. This was less than the values achieved in previous experiments (table 3.1.) but killing of transfected cells was effective. This difference is most likely explained by a lower tk activity, but this was not measured.

B95 $p53^-$ tk^+ cells were killed by low concentrations of GCV, but the mechanism of cell death may be important. For example, if cell death occurs only by apoptosis, then killing of $p53^-$ tumours may not be as effective as that of $p53^+$ tumours, because apoptosis is an immunologically silent event. The importance of the immune system has been described previously (section 1.11.5.). Therefore, if death of $p53^-$ cells occurs in a different way to $p53^+$ cells, studying $p53^-$ tumour models may be more suitable.

B95 $p53^-$ (tk^+ and tk^-) cells were treated with GCV for times of up to 48 hr, then genomic DNA was isolated. Changes in the integrity of DNA were investigated by an $\alpha[^{32}P]$-dCTP end labelling method (section 2.6.2.). Untreated cells and tk^- cells showed no evidence of DNA damage (figure 3.10.B.). However, laddering and smearing were seen in GCV treated tk^+ cells which indicates that apoptosis and necrosis occurred in parallel (figure 3.10.B).

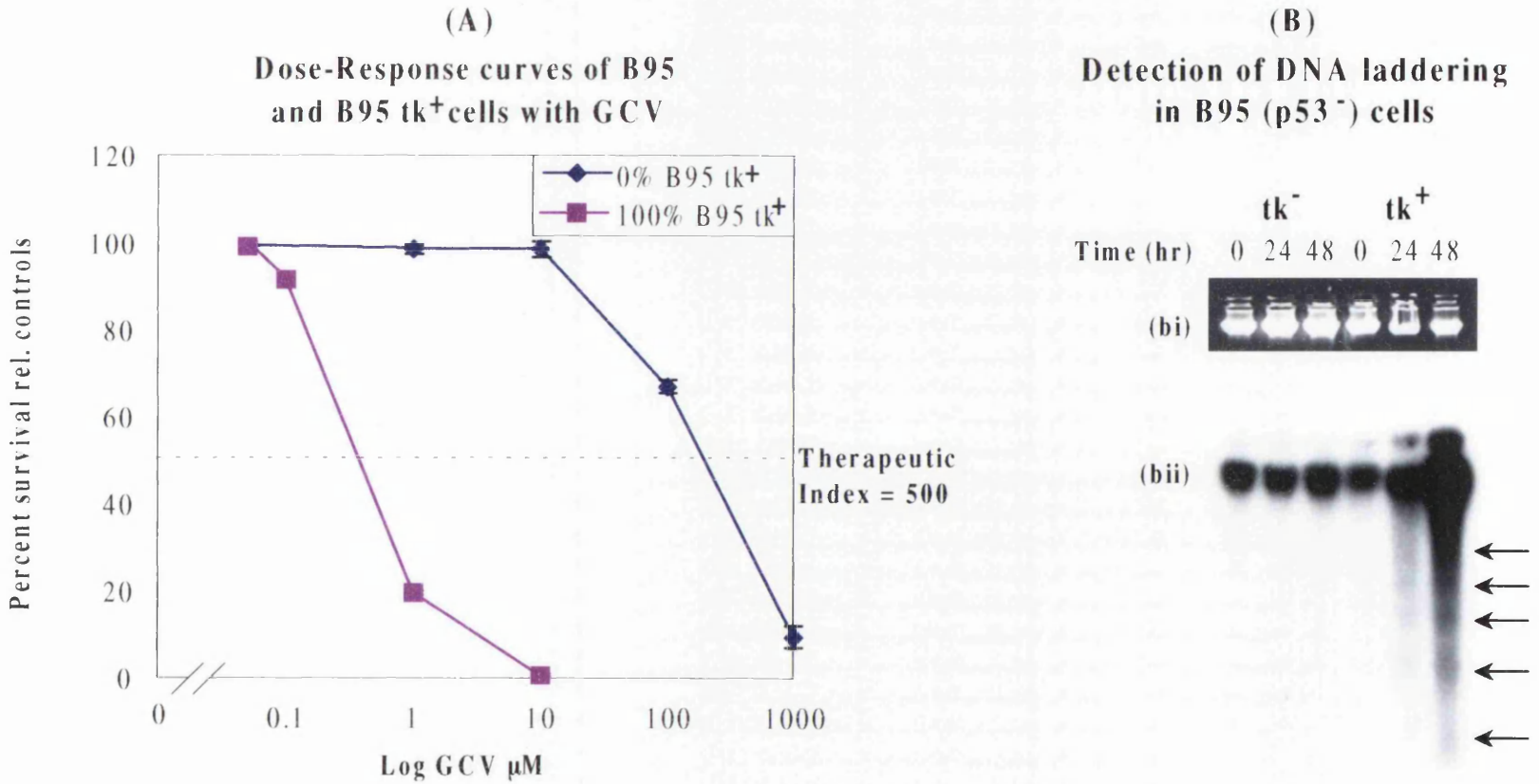


Figure 3.10. GCV induced tk cell death occurs by apoptosis and is independent of p53 status. B95 (p53-) were transfected with the tk gene. (**A**) Separate cultures of parental or transfected B95 cells were seeded (5 x 10^4 cells / well) in 24 well plates. 24 hr later cells were treated with GCV for 2 days. After this time cells were released with trypsin and aliquots of the suspension transferred to 96 well plates as described in section 2.2.7. (see Materials and Methods). Survival was determined using the MTT assay. (**B**) Presence of DNA fragmentation was determined by agarose gel electrophoresis of ^{32}P end labelled samples. Cultures of B95 or B95 tk^+cells were incubated with 20mM GCV for the times indicated above. Genomic DNA was isolated from these cells and free ends of DNA were labelled with a[^{32}P]-dCTP as described in section 2.6.2. (see Materials and Methods). **b(i)** UV illumination shows equal loading between samples. The gel was dried and exposed to autoradiographic film **b(ii)**. Only B95 tk^+ cells treated with GCV for 24 or 48 hr show evidence of DNA laddering (arrows) indicative of apoptosis.

GCV treatment of B95 $p53^-$ tk^+, L929 tk^+, CarB tk^+ and BHK tk^+ (section 3.3.4.) cells results in similar patterns of DNA degradation, suggesting that death involves the same mechanisms. It therefore seems likely that the p53 status will not affect the outcome of the tk / GCV strategy for cancer therapy.

3.3.8. Effective range of the tk / GCV bystander effect

It seems reasonable to conclude, from the experiments of the previous sections, that transfer of toxicity in the tk / GCV bystander effect occurs via gap junctions, by metabolic co-operation. Both donor and recipient cells must be able to form functional gap junctions. In addition, even when junctional communication is relatively low, high levels of tk activity can result in extensive bystander killing. Results have also demonstrated that GCV treatment of tk^- cultures mixed with 2.5% tk^+ cells can kill up to 65% of the cell population. This is an important observation but does not provide information on the effective range, the number of cell diameters, that toxicity can travel. It is likely that local or systemic delivery of a vector carrying the tk gene will lead mostly to transduction of cells near the injection site or near capillaries of solid tumours. Determining the distance toxicity can spread may, in conjunction with mixed culture survival assays, allow predictions of how effective treatments might be *in vivo*. The experiments below were designed to determine the effective range of tk / GCV bystander killing.

Colonies of tk^- cells were grown to increasing sizes and the effect of adding tk^+ cells, with GCV, on colony survival was measured. A full description of the assay is provided, step by step, in figure 3.11. It is important to note that tk^- cells were transfected with the βgal lineage marker ($tk^-\beta gal^+$) but are referred to as tk^- cells. Conversely, $tk^+\beta gal^-$ are referred to as

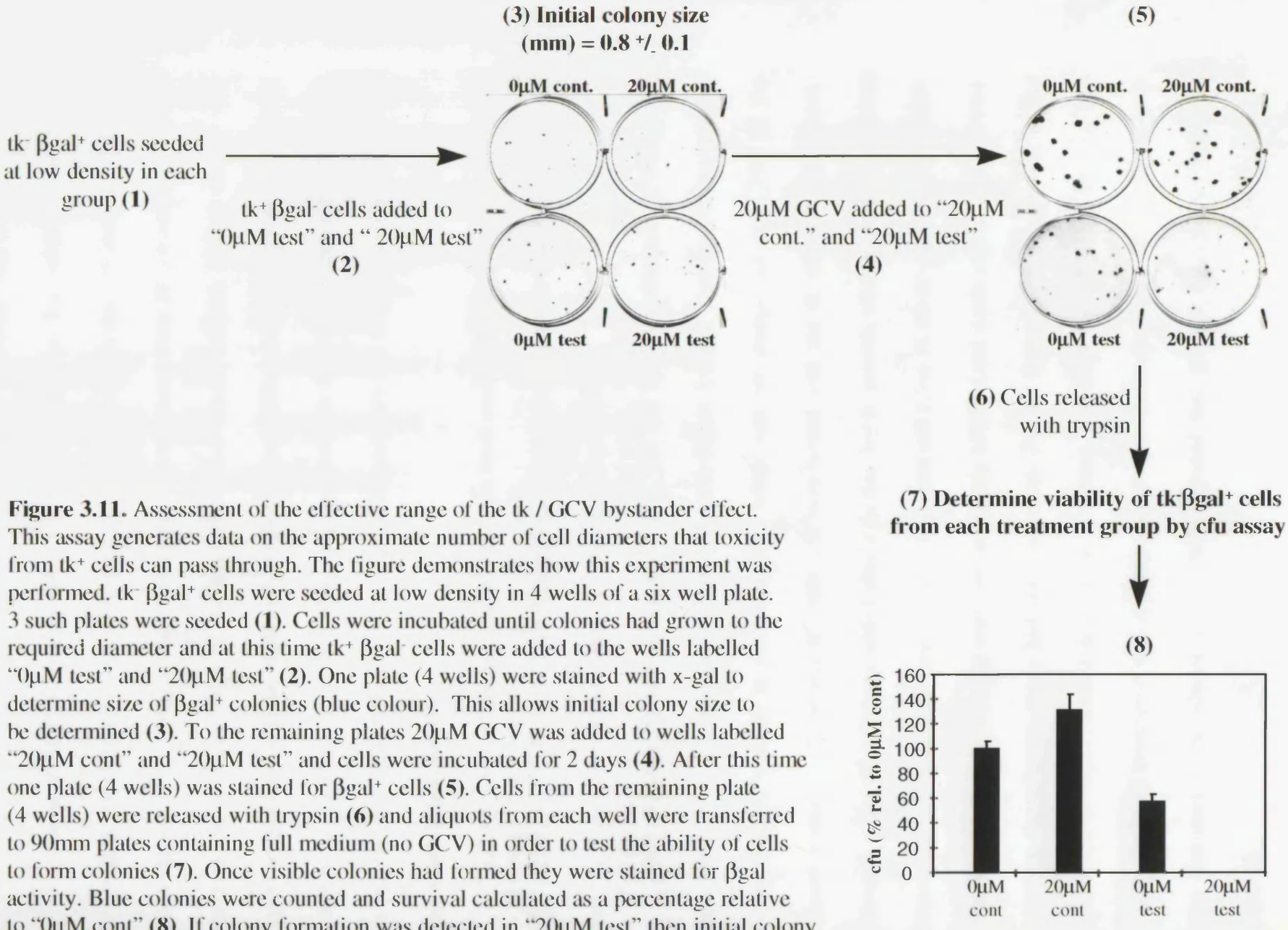


Figure 3.11. Assessment of the effective range of the tk / GCV bystander effect. This assay generates data on the approximate number of cell diameters that toxicity from tk+ cells can pass through. The figure demonstrates how this experiment was performed. tk- βgal+ cells were seeded at low density in 4 wells of a six well plate. 3 such plates were seeded **(1)**. Cells were incubated until colonies had grown to the required diameter and at this time tk+ βgal- cells were added to the wells labelled "0μM test" and "20μM test" **(2)**. One plate (4 wells) were stained with x-gal to determine size of βgal+ colonies (blue colour). This allows initial colony size to be determined **(3)**. To the remaining plates 20μM GCV was added to wells labelled "20μM cont" and "20μM test" and cells were incubated for 2 days **(4)**. After this time one plate (4 wells) was stained for βgal+ cells **(5)**. Cells from the remaining plate (4 wells) were released with trypsin **(6)** and aliquots from each well were transferred to 90mm plates containing full medium (no GCV) in order to test the ability of cells to form colonies **(7)**. Once visible colonies had formed they were stained for βgal activity. Blue colonies were counted and survival calculated as a percentage relative to "0μM cont" **(8)**. If colony formation was detected in "20μM test" then initial colony size exceeds the effective range of the tk / GCV bystander effect, whereas if colony formation is absent, initial colony size is within the effective range.

tk^+ cells. As described in figure 3.11, the presence of colony forming units in "20μM test" indicates initial colony size (step 3) exceeded effective range of toxicity from tk^+ cells.

Cultures of L929, CarB and BHK cells were investigated. Representative results for L929, which were controls since there is no bystander effect with this cell line, and BHK are shown (figure 3.12.). Initial average colony size was measured from 20 colonies and used to determine the effective range of the bystander effect (figure 3.12.A). Colony density was important since tk^+ cells can settle and grow amongst a diffuse colony which would result in spread of toxicity from tk^+ cells within a colony. Therefore the range of the bystander effect can only be determined when tk^+ cells settle at the periphery and this occurred when colonies were compact (see below).

In L929 control cultures, all colonies, even those small and diffuse, were unaffected by GCV treatment (figure 3.12.B) and this shows that any reduction of survival in BHK tk^- cells was not due to an artefact introduced by the experimental design.

BHK tk^- colonies were an average of 0.8-, 0.9- and 1.2-mm in diameter at the time of treatment and represent intervals of 24 hr of growth (figure 3.12.A). Treatment of the smallest colony (0.8mm) resulted in complete killing which was not unexpected since 75% of the colonies appeared diffuse and tk^+ cells will have settled with the tk^- colonies (see above). However, when tk^+ cells were added after a further day of growth, when the colony size was 0.9mm, and 90% appeared compact, GCV treatment still killed all the tk^- cells (figure 3.12.B). Colonies of 1.2mm in diameter were not completely inhibited and a minority of cells survived, indicating the effective range had been exceeded. Further increases in colony size, before addition of tk^+ cells, resulted in more tk^- survivors. Therefore the maximum distance that toxicity could be transferred was approximately 0.5mm (1mm diameter) and since BHK cells were estimated to be ≈20μm across, this suggested that passage through ≈25 cells was achieved.

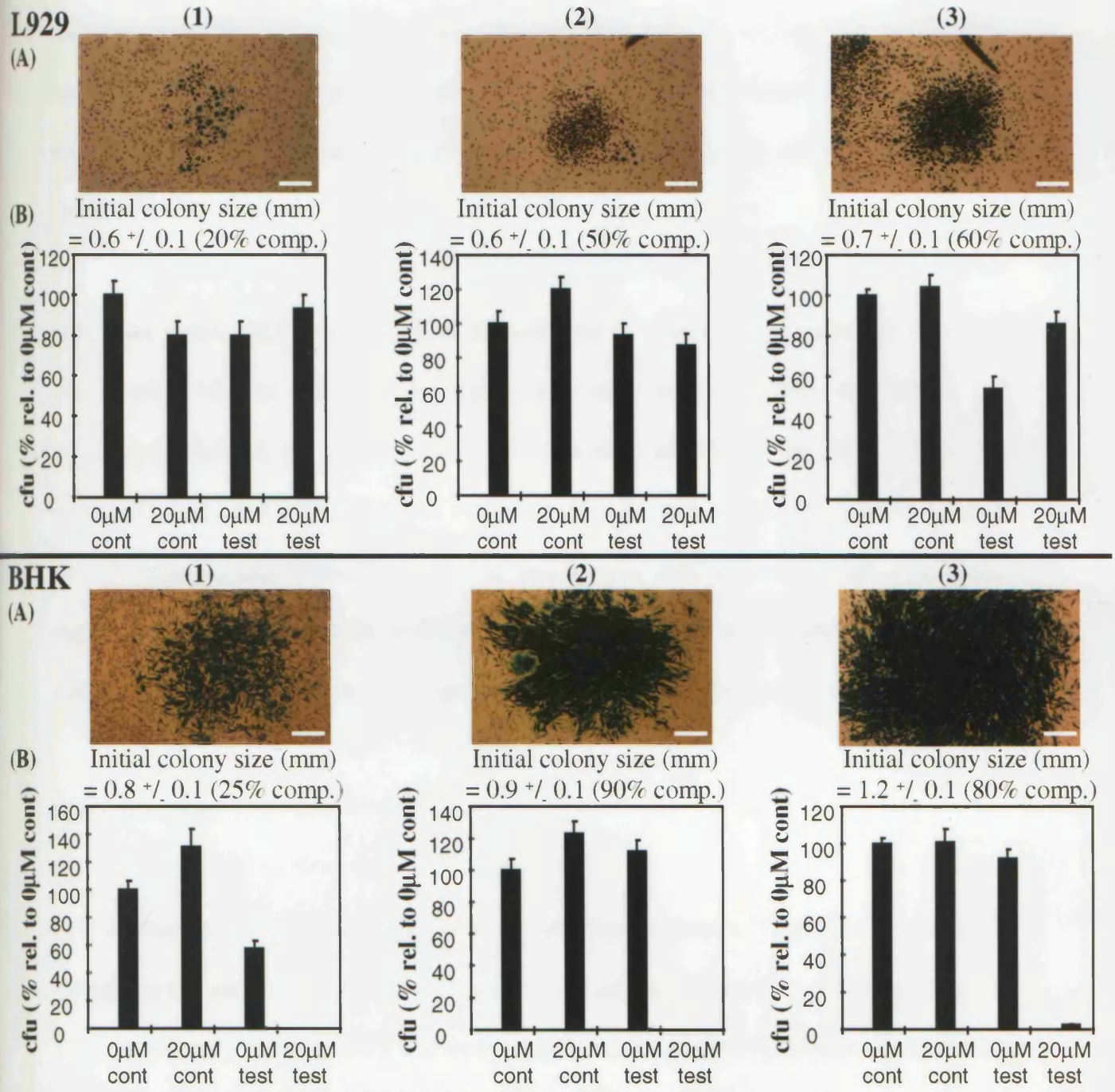


Figure 3.12. The effective range of the tk / GCV bystander effect in BHK cells is approximately 25 cell diameters. This experiment was performed as described in figure 3.11. Photomicrographs [L929 (A): BHK (A)] show representative initial colony sizes (bar 0.2mm). Values represent mean diameter +/- SE of 20 colonies (measurements taken from extremities). Colonies were scored as compact or diffuse (given as % comp.) and is a qualitative assessment. L929 (1) and BHK (1) are scored diffuse. L929 (3) and BHK (2) / (3) are scored compact. **(B)** Following the addition of tk^+bgal^- cells and GCV treatment, the ability of tk-bgal+ cells to form colonies was determined. Viability of L929 tk^-bgal^+ is not reduced compared to control. This demonstrates that there is no bystander effect in these cells. BHK tk^-bgal^+ colonies which are compact but under 1.2mm in diameter are completely inhibited after treatment. This demonstrates that in BHK cells colonies of up to 0.5mm radius (~25 cells) can be inhibited by addition of excess tk^+ cells combined with GCV treatment.

It is possible this result was distorted by tk^+ cells settling on, or within, tk^- colonies. If this did occur then toxicity would have been transferred to the colony from internal and external tk^+ cells and survival of tk^- cells would be reduced, even in large colonies. However, this did not occur and when colonies become sufficiently large, they survived (figure 3.12. BHK B-3).

In separate studies using the reverse strategy, CarB tk^+ colonies were grown, then tk^- cells were added. GCV treatment killed tk^+ and some tk^- cells leaving a section of the tissue culture plate without cells, showing that toxicity can spread effectively into the tk^- cell population. However, this was not seen in BHK cultures where tk^+ cells are killed, but residual tk^- cells formed a complete monolayer. Presumably, due to the faster growth rate and possibily migration rate of BHK cells, the area of tk^+ and tk^- cells that had been cleared, was rapidly repopulated. The limited data available from these experiments, are consistent with the estimate of 0.5mm as the effective range of the bystander effect, under these conditions.

3.4. Discussion

The nature of toxicity produced by tk^+ cells treated with GCV dictates the mechanism of transfer to cells without the suicide gene. The tk^+ cells studied here do not release a soluble toxic factor, such as cyclic GCV-MP or a cytotoxic cytokine. GCV-phosphates cannot cross the cell membrane and a more specialised transfer mechanism is required. This could involve direct cytoplasmic continuity through gap junctions or transfer based on release and uptake of vesicles containing cytoplasmic components. Such vesicular transfer is not normally detectable but may become more significant as cells degenerate during apoptosis (Cece et al, 1995; Hughes et al, 1996). If vesicles were involved, it is likely that transfer would occur by phagocytosis rather than fusion. This would result in vesicular contents ending up, not in the

cytoplasm, but in the lysomal compartment, still leaving a membrane(s) to cross to reach the cytoplasm. Despite such uncertainties, some mechanism based on vesicular transfer is often invoked as a possible basis of the bystander effect in the tk / GCV system (Freeman et al, 1993; Samejima and Mercelo, 1995).

The results here argue in favour of transfer of GCV metabolites via gap junctions for the following reasons. Cells that did not form gap junctions did not show a bystander effect (section 3.3.1.). Transfer of toxicity occurred before there was evidence of apoptotic degeneration (section 3.3.4.). The progress and extent of apoptosis (and presumably any vesicle formation) were similar in all cells examined, including those that showed no bystander effect. There was no detectable transfer of tk enzymic activity and it seems unlikely that released vesicles could selectively capture only particular cytoplasmic components. The bystander effect was absent in mixed cultures of cells that can form gap junctions and cells that cannot.

These data are consistent with a recent report (Hamel et al, 1996) that showed PA317 (mouse fibroblasts) tk^- cells can engulf cytoplasmic and nuclear material released from tk^+ cells but toxicity is transferred before these events occur. In addition these authors demonstrated that tk^+ cells preincubated with GCV for 2 hr had a diminished capacity to cause bystander killing of cells added subsequently, although phagocytosis still occurred.

The correlation between the tk / GCV bystander effect and gap junctions would be enhanced if it were possible to selectively modulate communication. However, chemical inhibitors are non specific (Nambu et al, 1989; Samejima and Mercuelo, 1995) and down regulation of Cx43 protein levels with antisense sequence had no detectable effect on GJIC of BHK cells (section 3.5.5.). Previous efforts in the laboratory to introduce communication into L929 and MCF-7 cells by expression of genes involved in gap junction formation have failed. For example, expression of E-cadherin in L929 cells (which express connexin 43 but not E-

cadherin) or connexin 43 in MCF-7 cells (which express E-cadherin but not connexin 43) had no effect on junctional communication. Gap junction formation in the cells studied here cannot be regulated by introducing or inhibiting one protein. Formation is more complex and a factor(s) that is presently unidentified may be involved.

It is possible to increase the level of communication in cells that are poorly coupled. However, increasing coupling in CarB cells by transfection with E-cadherin was not associated with an increase in bystander effect. This is in contrast to other reports that have shown a very strong correlation between level of communication and bystander effect (Fick et al, 1995; Elshami et al, 1996) and that increasing coupling of Hela cells by transfection with Cx43 can increase the bystander effect (Mesnil et al, 1996).

It is possible that the differences between the results presented here and these reports are due to differences in tk activity. In section 3.3.2. a strong correlation between level of tk activity and bystander effect was identified and this is consistent with data published by Chen et al (1995a). This suggests that in the cell lines studied here, high levels of tk activity prevent gap junctional communication from being a limiting factor in transfer of toxicity. This would be consistent with the high rates of transfer that can occur through gap junctions. For example, transmission of action potentials within excitable tissues, results in rapid changes (<msec) in ion concentrations through gap junctions while $HPRT^-$ cells, cultured with $HPRT^+$ cells, are able to maintain normal growth rates in medium containing aminopterin and hypoxanthine by acquiring all the purine nucleotides required for RNA and DNA synthesis via the junctional pathway (review, Pitts and Finbow, 1986 and see Pitts, 1971). The limiting factor may now be uptake of GCV by tk^+ cells or production of GCV-TP and incorporation into DNA in tk^- cells.

The reports showing a correlation between bystander effect and communication did not investigate the level of tk activity. If levels of tk activity were low, then concentration

gradients of GCV-phosphates will not be steep. In this case, an increased rate of exchange of small molecules between cells that are well coupled may facilitate bystander killing. Alternatively, if the number of gap junctions is sufficiently low it seems inevitable that transfer must become rate limiting, whether there is high or low tk activity. Increasing communication would then be an advantage. This may be the case for Hela cells where communication is increased by 40 fold (0.8 to 35 cell spread) with introduction of Cx43 (Mesnil et al, 1996).

Communication in tumorigenic cell lines is often reduced compared with normal cells taken from the same tissue (section 1.12.2.) and vectors that can achieve very high levels of tk expression will be required *in vivo*. If high levels of tk activity cannot be achieved, then delivery of a combination of the tk gene and genes involved in gap junction formation may enhance the bystander effect. Recently it was shown that tumour cure occurred more often when mice were inoculated with mixtures of C6tk$^+$ and C6tk$^-$ glioma cells that also had enhanced communication through transfection with Cx43 (Dilber et al, 1997).

It is likely, however, that below a certain threshold of tk activity, the bystander effect will be ineffective since there is a correlation between tk activity and bystander killing. It is also possible that low levels of tk activity will fail to lead to any cell death in communicating cultures since tk$^-$ cells may protect tk$^+$ cells (Wygoda et al, 1997). In this report, the authors suggested that cells may be tolerant to low levels of GCV-TP and if tk activity is low, toxic metabolites may become sufficiently dispersed and diluted within a communicating culture, to prevent killing.

An issue that should be considered if genes involved in gap junction formation are introduced to tumours is disruption of communication compartments. Use of the tk / GCV system to treat experimental tumours in animals is accompanied by little damage to normal surrounding tissue (Culver et al, 1992; Mullen, 1994). This is consistent with transfer by a gap junctional pathway. The body is divided into communication compartments. This results in

effective coupling of cells by gap junctions with in, but not between compartments. For example, cells in the dermal and epidermal communication compartments of new born mice are coupled (5 min dye spread: >500 or 25 cells, respectively) but only rarely does dye pass from one compartment to the other (Kam et al, 1986). It now seems likely that for tumour formation, cells must also form separate communication compartments (section 1.12.2.) and spread of toxicity from tk transfected tumour to normal cells will be minimised. Introducing genes such as connexin 43 may reduce such a barrier which will lead to spread of toxicity to normal tissues. Whether this would result in more extensive normal tissue damage would need to be investigated.

Chapter 4. Results - Analysis of the tk / GCV system *in vivo*

4.1. Introduction

Studies by others, using the tk / GCV system *in vivo,* have demonstrated the presence of a bystander effect and when only 10% of cells express the tk gene, tumours can be cured (Culver et al, 1992). These experiments were performed in immune competent animals and the bystander effect is likely to have been attributable to a combination of the immune system, damage to tumour vasculature and to transfer of toxicity by gap junctions. The immune system has been shown to play an important role in tumour cure (Vile et al, 1994b) but how important is junctional communication? Do bystander effects in culture correlate with tumour regression *in vivo*? If a correlation could be established then it might be possible to use cultured cells to evaluate the effectiveness of suicide gene delivery systems.

The results in chapter 3 showed that, in culture, L929 cells did not communicate and did not show a bystander effect, whereas CarB or BHK cells were coupled and bystander killing was seen. If a similar pattern arises in nude mice, where the role of the immune system is minimised, it would suggest a correlation between culture and *in vivo* bystander effect. This would reflect the importance of gap junctions in bystander killing and future efforts for the clinical application of this system should be focused on increasing tk expression or communication in tumours. However if a correlation is not seen, then ways of enhancing the immune response, or the use of other enzyme prodrug systems should be investigated.

4.2. Analysis of the tk / GCV bystander effect *in vivo*.

tk^+-βgal^- and tk^--βgal^+ cells from L929, CarB and BHK lines were used to study the bystander effect in nude mice. The presence of the βgal marker should allow tk^--βgal^+ cells to be located in tumours that fail GCV treatment. For simplicity tk^+-βgal^- and tk^--βgal^+ cells will be referred to as tk^+ and tk^-.

Initially the time course of tumour formation of tk^+ and tk^- cells was determined in pilot studies. CarB cells grew aggressively *in vivo*, requiring 4 days to form palpable tumours, while L929 and BHK cells required approximately 10 days. Based on tumour volume, tk^+ and tk^- cells, for each cell line, grew at a similar rate *in vivo*. This could not be pre-judged from the earlier *in vitro* studies, since cells with similar doubling times in culture may differ *in vivo* (Iwadate et al, 1996). These pilot studies also showed that intraperitoneal GCV injections did not cause noticeable discomfort to the animals and total body weight remained approximately constant.

To investigate the bystander effect *in vivo*, separate or mixed populations of tk^+ and tk^- cells were used to induce tumours in nude mice and the effect of GCV treatment on tumour volume was monitored.

From studies in culture (Chapter 3) and *in vivo* (Culver et al, 1992), it has been shown that a large proportion of cells can be destroyed when only 10% of the population are tk^+. Based on these observations, mixtures of CarB tk^+ and tk^- cells containing 0%, 5%, 10%, 50%, and 100% tk^+ cells were chosen. Only one PBS treatment group was included (100% tk^+). Pilot studies had already demonstrated that, in a limited number of animals, growth rates of tk^+ and tk^- tumours were similar, so it was unlikely that tumours derived from mixtures of these cells would have different growth rates. The results below show that the response of 5% tk^+ and 10% tk^+ groups was similar to GCV. In subsequent experiments with L929 and BHK

cells, the 5% tk^+ group was omitted. These omissions do not affect the interpretation of results but reduce the number of animals required. 8 to 10 animals were included in the groups for each cell mixture.

Nude mice were inoculated at two sites with suspensions containing 0% tk^+, 5% tk^+ (CarB only), 10% tk^+, 50% tk^+, or 100% tk^+ cells. Two groups of the 100% tk^+ animals were included, one for injection with GCV and one for injection with PBS. The animals were then left until tumours became palpable. Daily intraperitoneal injections of GCV (45mg / kg) or PBS (100% tk^+ group only) were initiated (termed day 1) and continued for 7 days. Tumours were measured during and after treatment, in two dimensions with callipers and the approximate tumour volume, based on the volume of a sphere ($^4/_3\ \pi r^3$), was calculated, then plotted against time (figure 4.1). The survival data were based on tumour volumes <500mm^3 and mice with tumours exceeding this limit were terminated.

L929, CarB and BHK tumours containing 0% tk^+ (100%tk^-) cells, treated with GCV, or 100% tk^+ (0% tk^-), treated with PBS, showed similar growth rates (figure 4.1.), which is consistent with culture data where tk^- cells are relatively insensitive to GCV. With L929 tumour bearing animals, GCV treatment did not result in a significant difference in survival between 0% tk^+, 10% tk^+ and 50% tk^+ groups where animals were terminated on days 10, 10 and 11, respectively (figure 4.1). L929 100% tk^+ (GCV) tumours regressed completely (no palpable lump) by day 5, but were not cured since all tumours recurred by day 22 and all animals were terminated at day 30 (figure 4.2.A.). These data, for L929 tumours, directly correlate with the culture data (section 3.3.1.) showing no bystander effect. The recurrence of 100% tk^+ tumours is investigated further in section 4.3.

Animals with tumours formed from CarB 5% tk^+ or CarB 10% tk^+ cell mixtures showed an increase in survival of 9 days (to day 14), compared to those with the 0% tk^+ control tumours, which where were terminated on day 5. When half the inoculation consisted

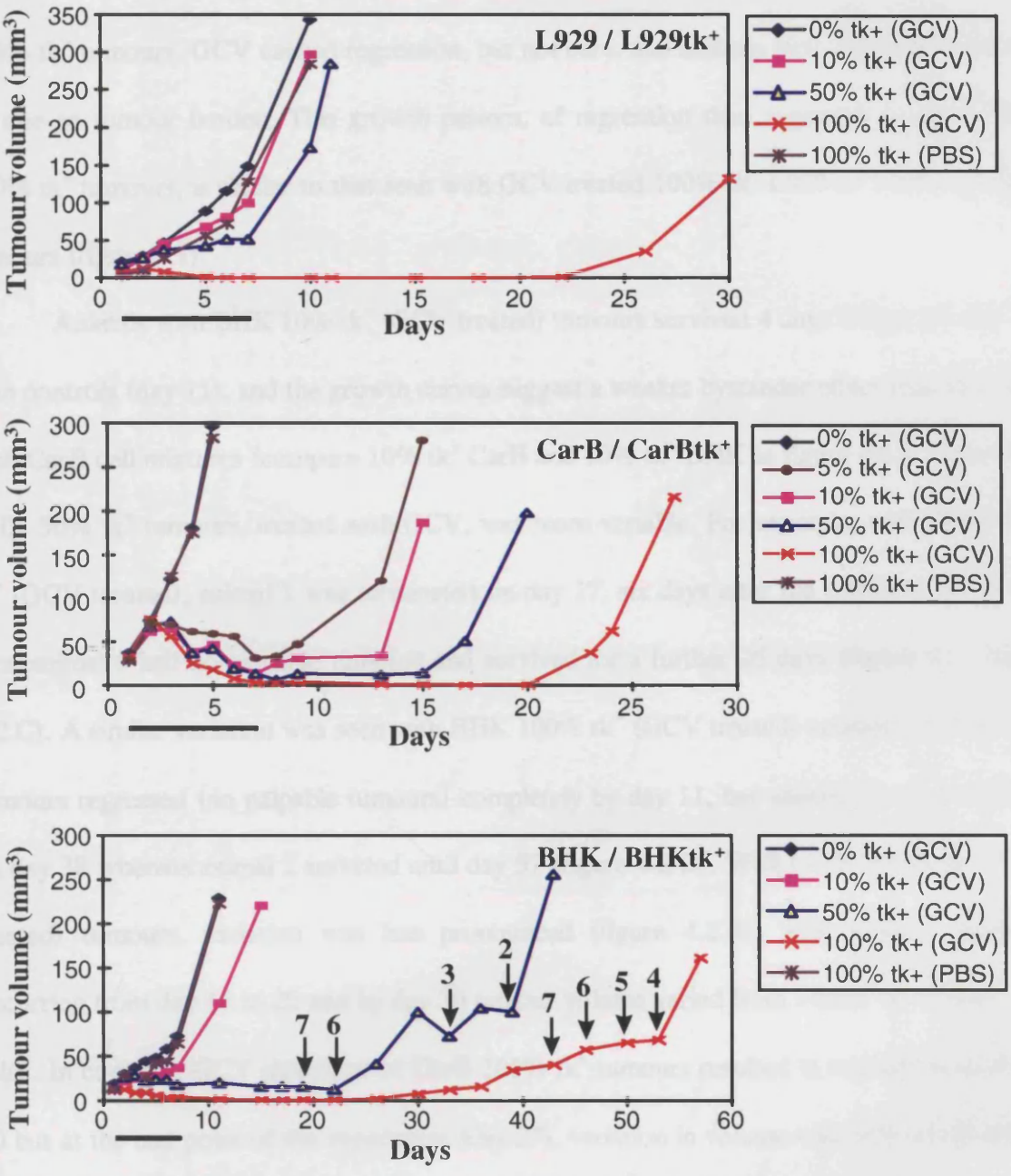


Figure 4.1. GJIC plays a role in the tk / GCV bystander effect in vivo. Tumours were induced in nude mice by injecting separate or mixed populations of tk^+ and tk^- cells subcutaneously into each flank (10^5 cells / site). The study invo lved ten animals per group for CarB and 8 animals per group for L929 and BHK tumours. After 4 to 10 days tumours had grown to approximately 3 to 5 mm in diameter. At this time intraperitoneal injections of GCV (45mg / kg, once daily) were initiated and continued for 7 days (bars). Approximate tumour volumes were calculated from the average of two measurements ($^4/_3\Pi r^3$). Animals were removed from the study when the total tumour burden exceeded $500mm^3$. In the BHK study animals with tumours containing 50% tk^+ or 100% tk^+ cells (treated with GCV) regrew at different times. The arrows indicate when animals were removed from the study due to excessive burden and the values indicate the number of animals remaining in the group. These data demonstrate that no bystander effect was seen in tumours which lack GJIC (L929), whereas tumours which communicate (CarB and BHK) did show an effect.

of CarB tk^+ cells, animals survived until day 20 (figure 4.1). The tumour growth curves (figure 4.1.) indicate a bystander effect *in vivo*, which correlates with that seen in culture. With CarB 100% tk^+ tumours, GCV caused regression, but not cure, and animals were terminated on day 27 due to tumour burden. This growth pattern, of regression then regrowth in these CarB 100% tk^+ tumours, is similar to that seen with GCV treated 100% tk^+ L929 or 100% tk^+ BHK tumours (figure 4.1).

Animals with BHK 10% tk^+ (GCV treated) tumours survived 4 days longer (to day 15) than controls (day 11), and the growth curves suggest a weaker bystander effect than that seen with CarB cell mixtures (compare 10% tk^+ CarB and 10% tk^+ BHK in figure 4.1.). Growth of BHK 50% tk^+ tumours, treated with GCV, was more variable. For example, with BHK 50% tk^+ (GCV treated), animal 1 was terminated on day 17, six days after the controls, but at this time animal 6 had no palpable tumours and survived for a further 26 days (figure 4.1.; figure 4.2.C). A similar variation was seen with BHK 100% tk^+ (GCV treated) tumours, where most tumours regressed (no palpable tumours) completely by day 11, but animal 5 was terminated on day 38 whereas animal 1 survived until day 57 (figure 4.2.D). With L929, 100% tk^+ (GCV treated) tumours, variation was less pronounced (figure 4.2.A), with tumour regrowth occurring from day 15 to 22 and by day 30 tumour volume varied from $14mm^3$ to $450mm^3$ (32 fold). In contrast, GCV treatment of CarB 100% tk^+ tumours resulted in regression until day 20 but at the end point of the experiment (day 27), variation in volume was only 6 fold (figure 4.2.B).

The irregularity of growth in some groups did not correlate with initial size. For example, with L929 100% tk^+ (GCV treated) tumours, the initial volume for animal 7 was $28mm^3$ but at day 30 was $14mm^3$, while over the same period burden of animal 6 increased from $0.5mm^3$ to $449mm^3$ (figure 4.2.A). Variations in growth rate of tumours within the same

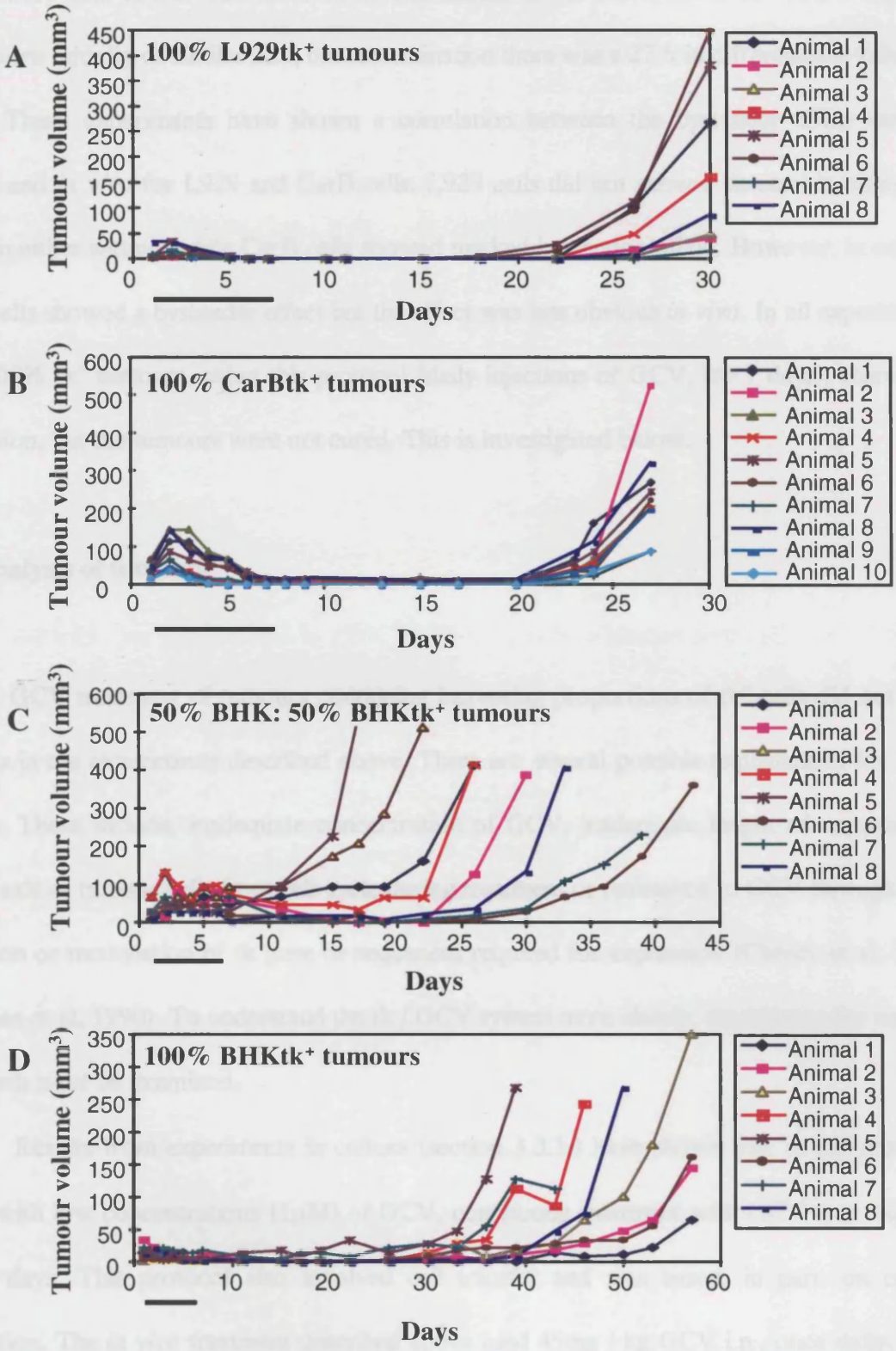


Figure 4.2. Recurrence of GCV treated tk tumours occurs at varying times. Subcutaneous tumours were induced as described in figure 4.1. Tumours containing 100% CarB tk$^+$ cells regrew at very similar times (Day 20) whereas regrowth (increases in tumour volume after GCV treatment) within the other three groups varied (L929 100% tk$^+$, day 15 to 22; BHK 50% tk$^+$, day 7 to 22; BHK 100% tk$^+$, day 17 to 39). Time of recurrence did not correlate with tumour volume at the initiation of GCV treatment.

animal were also noted. The tumours of one animal in the BHK 100% tk^+ (GCV treated) group were initially of similar size, but at termination there was a 27 fold difference in volume.

These experiments have shown a correlation between the bystander effect seen in culture and *in vivo* for L929 and CarB cells. L929 cells did not show a detectable bystander effect in either setting, while CarB cells showed marked bystander killing. However, in culture BHK cells showed a bystander effect but the effect was less obvious *in vivo*. In all experiments with 100% tk^+ tumours, using this protocol (daily injections of GCV, for 7 days), there was regression, but the tumours were not cured. This is investigated below.

4.3. Analysis of tumours

GCV treatment of tumours containing increasing proportions of tk^+ cells did not cure animals in the experiments described above. There are several possible explanations for these results. These include, inadequate concentration of GCV, inadequate length of exposure to GCV, exit of tumour cells from cell cycle during treatment or resistance to GCV through loss, mutation or methylation of tk gene or sequences required for expression (Christy et al, 1982; Moolten et al, 1990). To understand the tk / GCV system more clearly, the reasons for tumour regrowth must be examined.

Results from experiments in culture (section 3.3.1.) have shown that to kill most tk^+ cells, with low concentrations (1μM) of GCV, continuous treatment with GCV was required for 5 days. This protocol also involved cell transfer and was based, in part, on colony formation. The *in vivo* treatment described above used 45mg / kg GCV i.p., once daily, for 7 days. In humans, the half life of GCV is 2-4 hr, and the peak plasma concentration, after i.v. infusion of 10mg / kg GCV, daily, is ≈20μM (Markham and Faulds, 1994). Peak concentration in mice can be expected to be higher, due a 4.5 fold higher dose and a different

method of delivery (i.p. injection produces higher peak plasma concentration of prodrug than i.v. injection; Huber et al, 1995), but the half life may be different. In addition, the concentration of GCV in the tumour might be expected to be lower than the plasma concentration. tk^+ cells may be exposed to toxic doses of GCV for relatively short times and this suggests tumour recurrence may be due to an inadequate or an unsustained concentration of GCV in the tumour, with the consequent survival of the tk^+ cells.

To examine the tk activity remaining in the tumours, the tk assay (section 2.4.3.) was calibrated and activity was shown to be proportional to the percentage of tk^+ cells in mixed cultures of tk^+ and tk^- cells (figure 4.3.A). Lysates were prepared from tumour samples and tk activity measured (figure 4.3.B).

Activity (per mg protein) in BHK 100% tk^+ tumours treated with GCV or PBS was close to that of tk^+ cultured cells. This observed tk activity was consistent with the presence of the tk gene seen in Southern blot analysis (figure 4.4.Aii) and a similar GCV sensitivity, relative to BHK tk^+ cells, when tumour cells were returned to culture (figure 4.4.B). This suggests that complete tumour cure failed because cells were not cycling, or the concentration of GCV was too low, and this suggestion was supported by subsequent experiments (section 6.4.) where GCV treatment using the same regime above for 14 (instead of 7) days can cure tumours expressing the tk gene.

The low levels of tk activity of tumours derived from the 10% tk^+ or 50% tk^+ L929, CarB and BHK cell mixtures suggests that more tk^- cells survived than tk^+ (figure 4.3.) and that bystander killing (in the CarB and BHK tumours) is less efficient than direct killing of the tk^+ cells. It was hoped that these suggestions would be supported by x-gal staining of tumours, however, staining of replicate samples was not consistent, and it would be unwise to place much emphasis on these histological results.

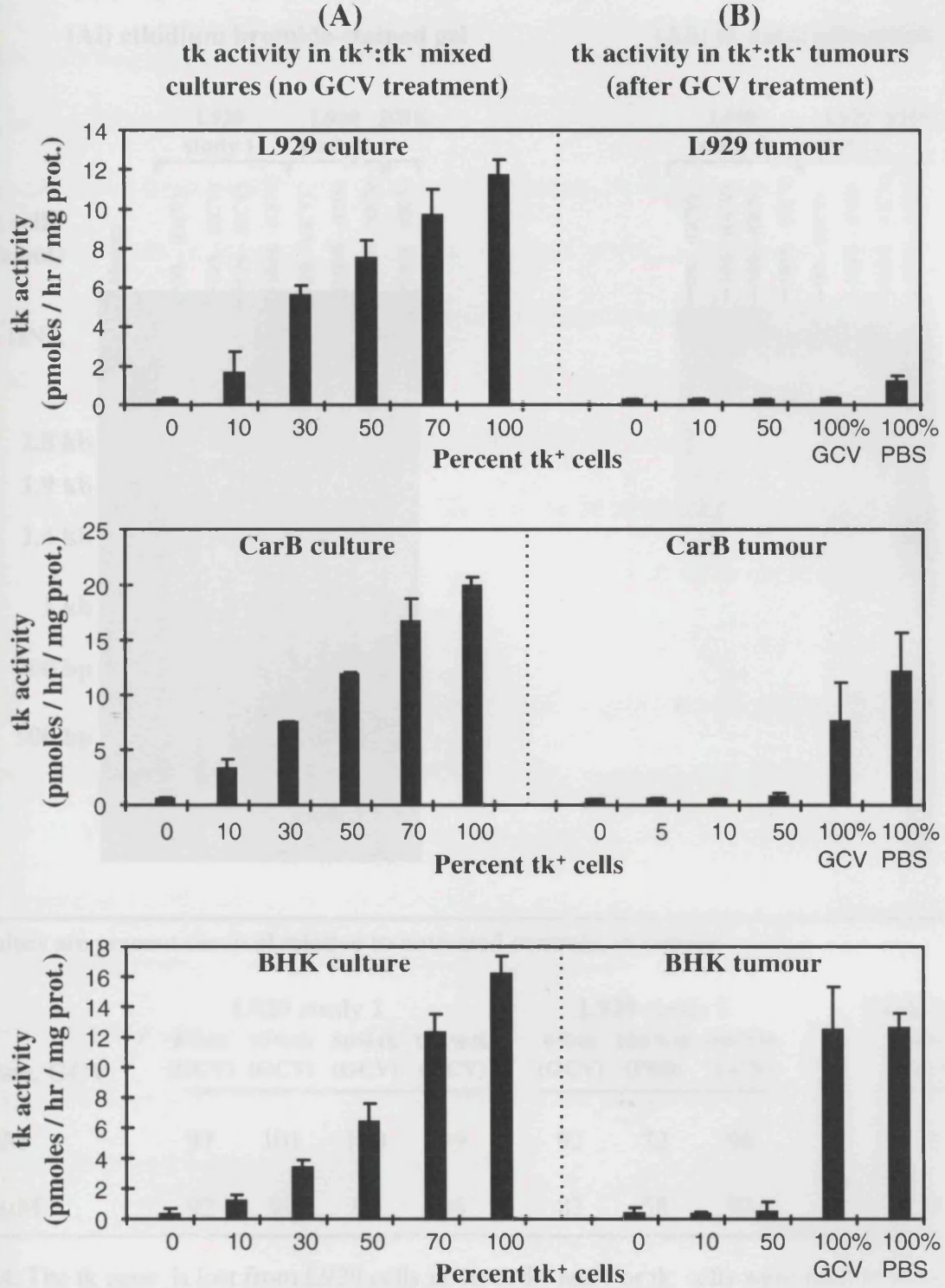
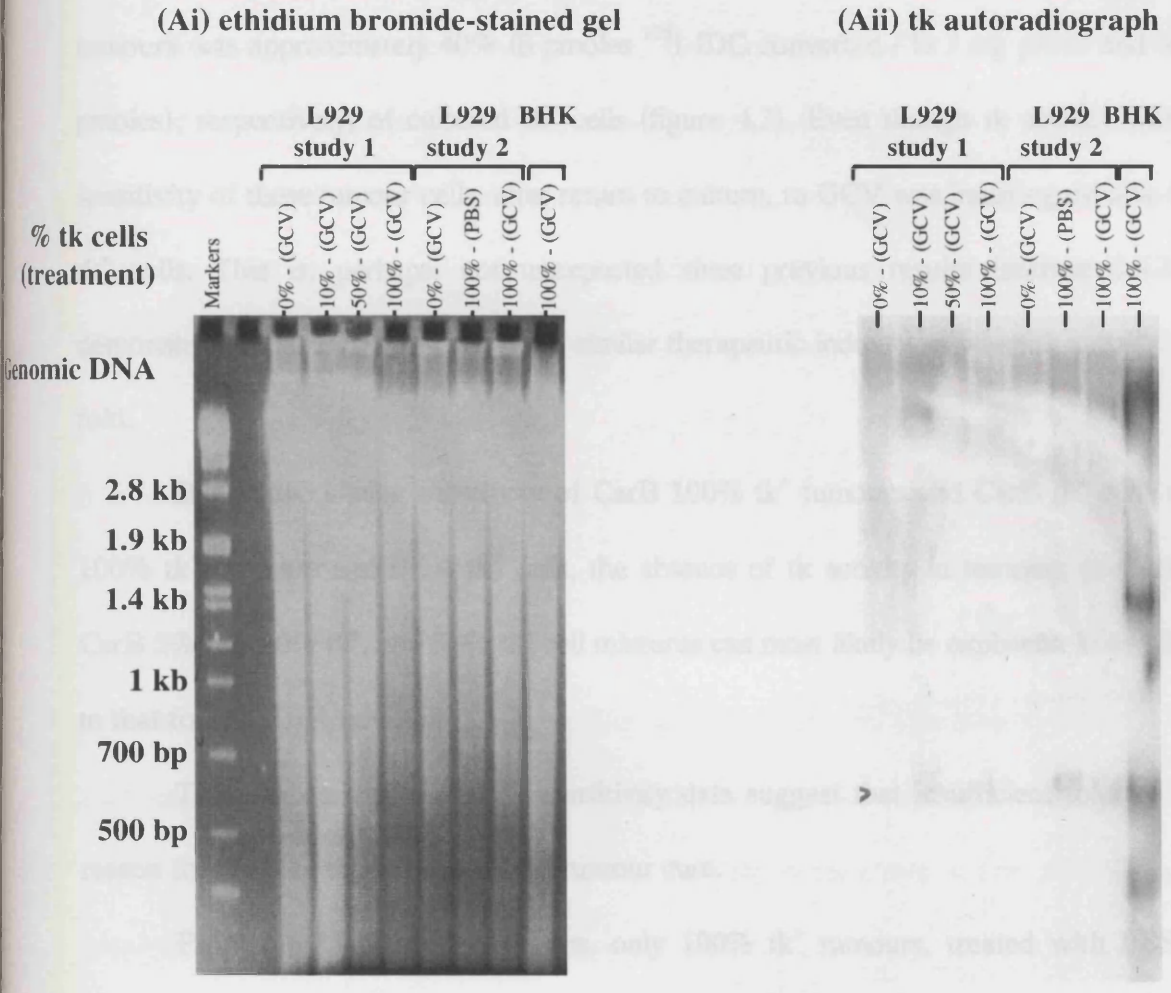


Figure 4.3. Analysis of tk enzyme activity in cells before and tumours after GCV treatment. **(A)** Separate or mixed cultures of tk^+ and tk^- cells were seeded (1 x 10^6 / cells) in 90mm plates at the ratios indicated above. 24 hr later cells were harvested and tk activity determined as described in section 2.4.3. Data represent mean +/- SEM of two independent experiments, each performed in duplicate.This demonstrates that percentage of cells with tk expression correlates to activity. **(B)** In all treatment groups tumours were not cured (see figure 4.1.). Tumours were removed, snap frozen and protein lysates prepared from tumours from each group. tk activity was analysed. Data represent mean +/- SEM activity from 3 tumours, each analysed in duplicate experiments.



(B) Values are percent survival relative to untreated controls, in culture

	L929 study 1				L929 study 2			BHK study
Conc. GCV	0%tk (GCV)	10%tk (GCV)	50%tk (GCV)	100%tk (GCV)	0%tk (GCV)	100%tk (PBS)	100%tk (GCV)	100%tk (GCV)
1μM	97	101	100	99	92	72	96	1
10μM	92	99	92	96	87	58	93	0

Figure 4.4. The tk gene is lost from L929 cells in vivo. tk^+ and / or tk^- cells were used to induce tumours as described in figure 4.1. After treatment with GCV tumours regrew. Tumours were removed and presence of the tk gene analysed by **(A)** Southern blot analysis or **(B)** sensitivity to GCV in culture. **(A)** 15μg of genomic DNA was digested with SstI and SmaI restriction enzymes, which releases a 700bp fragment of the tk gene. DNA was separated by agarose gel electrophoresis **(Ai)** before transfer to Hybond N^+ membrane. (Aii) shows the autoradiograph after probing the membrane with full length ^{32}P labelled tk cDNA (see Materials and Methods). **(B)** L929 or BHK cells were isolated from tumour samples and grown in culture. Cells were seeded at 1 x 10^5 and 5 x 10^4 cells / ml, respectively, in 24 well plates. 24 hrs later cells were treated with GCV for 2 days. Survival was assayed using standard protocol (see Materials and Methods) and compared to untreated controls. Data represent mean of one experiment, performed in triplicate.

Analysis of CarB 100% tk^+ tumours revealed that tk activity in GCV and PBS treated tumours was approximately 40% (8 pmoles ^{125}I-IDC converted / hr / mg prot.) and 60% (12 pmoles), respectively, of cultured tk^+ cells (figure 4.3). Even though tk activity was lower, sensitivity of these tumour cells, after return to culture, to GCV was indistinguishable to CarB tk^+ cells. This is, perhaps, not unexpected since previous results (section 3.3.2.) have demonstrated that BHK tk^+ cells had a similar therapeutic index even when tk activity varies 5 fold.

Due to the similar sensitivity of CarB 100% tk^+ tumours and CarB tk^+ cells or BHK 100% tk^+ tumours and BHK tk^+ cells, the absence of tk activity in tumours produced from CarB 5% tk^+, 10% tk^+, and 50% tk^+ cell mixtures can most likely be explained in a similar way to that for the BHK tumours.

These tk activity and GCV sensitivity data suggest that insufficient toxicity was the reason for the lack of BHK and CarB tumour cure.

From the L929 tumour groups, only 100% tk^+ tumours, treated with PBS, had a detectable (<10% activity of cultured L929 tk^+ cells) level of tk activity (figure 4.3.). These observations were consistent with analysis of the sensitivity of L929 tumour samples to GCV, after return to culture, where 10μM GCV, a concentration that kills all cultured L929 tk^+ cells, killed 42% of L929 100% tk^+ (PBS treated) cells and had no significant effect on the survival of GCV treated tumour groups (figure 4.4.B.).

Loss or reduced tk activity of L929 tumours may have occurred from disruption of the tk gene and / or promoter, or from methylation of the promoter. The absence of the tk gene in all GCV treated L929 tumours and the presence of low levels of the tk gene in PBS treated L929 100% tk^+ tumours suggests that gene loss caused resistance to GCV, hence caused recurrence in L929 100% tk^+ (GCV treated) tumours (figure 4.4.Aii.).

Together the results from these studies have shown that tumours expressing the tk gene were not cured with daily treatment of GCV for 7 days, and it is likely that this was due to insufficient exposure to GCV, and / or resistance to GCV caused by loss of the tk gene. Stability of the tk gene varied between the cell lines studied, for example, loss occurred within 20 days in L929 tumours but there was no significant reduction in expression after 60 days in BHK tumours.

4.4. Discussion

The intention of the experiments in this chapter was to determine whether a correlation exists between the levels of bystander effect seen in culture and that seen *in vivo*. If there was such an association, the culture model would be a useful tool for evaluating important parameters for this form of cancer therapy, and the requirement for *in vivo* tests may be reduced.

The absence of a bystander effect in L929 cells, in culture, correlates with lack of killing L929 tk^- cells *in vivo*. These cells do not communicate in 2 dimensions (in culture) and from the experiments in this chapter, it appears that cells do not become coupled in a 3 dimensional setting (*in vivo*). This observation supports recently published results (Ishii-Morita et al, 1997) which showed that some cell types can be killed a bystander effect *in vivo* (bystander sensitive) while others cannot (resistant). Mixing sensitive and resistant cells in culture, or as tumours, *in vivo*, also prevented a bystander effect. When these cells were investigated in culture, metabolic co-operation only occurred between bystander sensitive tk^+ and tk^- cells (Ishii-Morita et al, 1997). These culture data also support those in section 3.3.6.

Although it is anticipated that the majority of tumours will be coupled (Yamasaki, 1990), albeit at lower levels than corresponding normal tissues, it would be undesirable to use

the tk / GCV system when cells do not form gap junctions, since there would be no bystander effect. For example, tumours derived from some nerve cells and lymphocytes would be poor choices for this therapy since they are known not to form gap junctions. Consistent with this expectation, the tk / GCV combination does not lead to bystander killing in lymphocyte cultures (Tiberghien et al, 1993; Rogers et al, 1996). However, analysis of cells in culture that have been explanted from tumours may produce artefactual results. For example, MCF-7 breast cancer cells are uncoupled as a monolayer but when grown in 3 dimensions, on a collagen based matrix, the cells communicate, albeit to a limited extent, as judged by Lucifer Yellow microinjection (Quarrie, personal communication). Low levels of transfer measured by dye transfer (2 minutes) can permit extensive nucleotide exchange over periods of several hours (Pitts and Kam, 1985). To evaluate patterns of communication in human tumours, it may be necessary to explant tumour segments and measure coupling in 3 dimensions by Lucifer Yellow microinjection into cells in the tissue. Levels of communication in the dermis and epidermis of newborn mice have been determined in this way (Kam et al, 1986). This is not to say that junctional communication will be the only consideration in determining whether the tk / GCV system should be used on a particular tumour. The importance of the immune system has been stated several times before (see section 1.11.5.).

From the studies here it is clear that there are some variations between the bystander effects seen in culture and those seen *in vivo*. Comparing CarB with BHK cells, the extent of bystander killing in culture is similar but *in vivo* the effect in BHK tumours is weaker. This might be accounted for by a difference in growth rates. For example, in culture CarB cells have a longer doubling time than BHK cells, but *in vivo* CarB cells grow aggressively which may make them more susceptible to killing by a system that relies on DNA damage. In support of this suggestion, Iwadate et al (1996) demonstrated that GCV treatment of animals with established, slow growing 9L tk^+ tumours, increased survival by 7 days compared to controls,

whereas in parallel experiments, with fast growing 9L tk^+ tumours, survival was increased by >60 days. Therefore the tk / GCV system is likely to be most effective for fast growing, aggressive tumours and in those with slower growth rates, the introduction of cytokine genes, to stimulate the immune system, or the use of different enzyme prodrug systems, should be considered.

In the experiments described here, all tumours recurred, but those derived from CarB tk^+ or BHK tk^+ cells, retained high levels of tk activity. This suggests that cells were out of cell cycle or were insufficiently exposed to GCV. Support for this suggestion comes from Osaki et al (1994) who showed extending or increasing the dose of GCV can lead to tumour cure.

In some cases prolonging the treatment regime does not result in tumour cure (Cool et al, 1996). In these situations resistance to GCV may be acquired. It is possible that this arises from hypermethylation of the promoter (Veelken et al, 1996) or by gene inactivation, by loss or mutation. *In vivo*, L929 tk^+ tumours become resistant to GCV within 15 to 20 days of implantation. This is unlikely to result in resistance when tumour cells are targeted by recombinant adenovirus since GCV treatment is likely to commence with 24 hr of infection (Chen et al, 1995b). However transduction of tumours by recombinant retroviral particles generated from vector producer cells (vpcs; see section 1.15.1.) is optimal after 7 days (Culver et al, 1994) and the stability of the tk gene in the producer cell line will need to be assessed.

More of a concern, are treatments that may require long term tk stability. For example, transduction of lymphocytes to avoid graft versus host disease (GVHD; section 1.4.1.) or combined expression of *mdr1* and tk genes in protection of stem cells against chemotherapeutic agents (section 1.4.2.). If resistance to tk arises in these instances then GVHD or cancer cells expressing multiple drug resistance protein may result. A potential way to avoid cellular resistance to prodrugs is to incorporate multiple suicide genes, a strategy which has proved effective in culture, where introduction of multiple copies of the tk gene to

murine sarcoma cells correlated with reduced frequency of GCV resistant clones (Moolten et al, 1992b). Methods of achieving expression of several genes within cells will be considered further in chapter 7.

Chapter 5. Results - Analysis of tp / DFUR and cd / FC enzyme / prodrug systems.

5.1. Introduction

For enzyme / prodrug systems to function in cancer gene therapy the characteristics of each strategy will have to be appropriate for the target tumours. For example, the tk / GCV system requires cells to be cycling and, for a bystander effect, gap junctional communication. Enzyme / prodrug systems that do not rely on gap junctions will be better suited to some tumours, such as lymphomas. The pathways of the tp / DFUR and cd / FC systems produce FU as a common toxic metabolite. This base analogue can cross the cell membrane and using the cd / FC combination, bystander effects that are independent of cell-cell contact, have been demonstrated (Hirschowitz et al, 1995; Lan et al, 1996). FU is subsequently converted to phosphorylated products that are not be able to cross the cell membrane (section 1.10.2.; 1.10.3.; figure 1.2.). If this conversion operates at a high rate then the level of membrane permeable metabolites may be reduced. However, the presence of gap junctions may allow additional, and perhaps more rapid transfer of toxic compounds, which may enhance the bystander effect.

The effectiveness of the tk / GCV and cd / FC systems have been compared (Rogers et al, 1996; Hoganson et al, 1996; Trinh et al, 1995) and in each report the therapeutic index achieved was greater with the cd system than with the tk system. It is important to determine whether the cd / FC system is always superior because it will be desirable to choose the most effective system for cancer therapy. The tp / DFUR combination has not been compared to any other enzyme / prodrug system.

This chapter investigates the efficacy of the tp / DFUR and cd / FC systems in a way that enables them to be compared to the tk / GCV strategy. The potential role of gap junctions in transfer of toxicity in the tp / DFUR bystander effect is also examined.

5.2. Growth characteristics of cell lines transfected with the tp or cd genes.

Introduction and expression of the tp gene to L929, CarB and BHK cells was not achieved as easily as with the tk gene. Transfection of L929 cells with a plasmid carrying the tp gene and Neo^R marker produced G418 resistant colonies, but only a fraction (≈ 15%) were sensitive to 1μM DFUR, a concentration that had no effect on survival of parental cells. Transfection of CarB and BHK cells, by this approach, did not produce any colonies that were sensitive to 1μM DFUR. For comparison, after transfection of these cell lines with the tk gene, >50% of G418 resistant colonies were sensitive to GCV.

Due to the difficulty in achieving tp expression in CarB and BHK wild type cells, alternative approaches, including subcloning of the tp gene into different plasmids and the use of retroviral transduction, were used to produce double transfected CarB tk^+-tp^+ or BHK tk^+-tp^+ cells (from CarB tk^+ or BHK tk^+ cells, section 3.2.2.). These double transfected cells would allow the same cells to be used to study the tp / DFUR and the tk / GCV systems, and for analysis of the potential advantages of combining the tk and tp systems (section 6.3.). For simplicity, the CarB tk^+-tp^+ or BHK tk^+-tp^+ clones that were used are termed CarB tp^+ or BHK tp^+, in this chapter.

For analysis of the cd / FC system, L929 and BHK wild type cells were transfected with the cd gene (section 2.2.3.) and G418 resistant clones that were sensitive to 1000μM FC, were expanded.

The population doubling time of a selected BHK tp^+ clone was similar to that of the wild type cells, but the rate of proliferation of an L929 tp^+ clone was decreased while that of a CarB tp^+ clone was increased (Table 5.1.; figure 3.1.). The variations that were seen can most likely be explained by clonal differences and will be considered in the results of the analyses in section 5.4. The levels of GJIC of these tp^+ clones were measured by Lucifer Yellow microinjection, and found to be not significantly different (t-test, $p>0.05$) from those of wild type cells (Table 5.1.; figure 3.1.).

The population doubling times of the L929 cd^+ and BHK cd^+ selected clones were not significantly different (t-test, $p>0.05$) to the respective wild type cells (Table 5.2.; figure 3.1.). GJIC was not measured in these clones since studies which were dependent on the level of coupling were not performed.

5.2.2. Analysis of the tp / DFUR and cd / FC bystander effect in culture.

In order to make a comparison between the tk / GCV, tp / DFUR and cd / FC systems, analyses of the latter systems were performed in parallel experiments to those used to analyse the tk / GCV strategy (section 3.3.1.; section 2.2.7.). Treatment of tp^+ cell lines with DFUR, at concentrations that had no effect on the survival of parental cells, led to almost complete cell death (figure 5.1.A.). In mixed cultures of tp^+ and tp^- cells, bystander killing of tp^- cells was seen in cell lines with (CarB or BHK) or without (L929) GJIC. For example, when the culture contained 10% tp^+ cells, 1μM DFUR killed 30% of L929 cells or 80% of CarB cells, while 10μM DFUR killed >90% BHK cells (figure 5.1.A.). These concentrations caused <15% cell death in untransfected cultures (figure 5.1.A.). The bystander effects in this system did, however, appear weaker than those in the tk / GCV system and this can be seen more clearly by calculating the IC50s from the survival curves of each mixed culture (figure 5.1.B.;

Table 5.1.). For example, in cultures containing 10% BHK tp^+ cells, there was a 12 fold decrease in the IC50 with respect to DFUR, while in those with 10% BHK tk^+ cells there was a 290 fold reduction in the IC50 with respect to GCV (figure 5.1.; Table 5.1.; Table 5.3.). Similar differences were seen between the therapeutic indices of these systems and this will be considered further in section 5.5.

From the cell lines studied, the weakest bystander effect, in the tp / DFUR system, was seen using mixed cultures of L929 tp^+ cells, where 10% tp^+ cells reduced the IC50 by 6 fold compared with untransfected cells (Table 5.1.; Table 5.3.). It is possible that the presence of gap junctions in the CarB and BHK cell lines enhances bystander killing by transfer of phosphorylated toxic metabolites to tp^- cells and this suggestion is investigated further in section 3.4.

Using the cd / FC system, L929 cd^+ and BHK cd^+ cells were almost completely killed by 1000μM FC, a concentration of prodrug that did not affect the survival of parental cells. In mixed cultures of 10% cd^+ and 90% cd^- cells, 1000μM FC killed ≈70% of the L929 culture and ≈50% of the BHK culture. These bystander effects are seen more clearly as reductions in IC50 compared with untransfected cultures (figure 5.2.; Table 5.2.). Cultures containing 10% L929 cd^+ cells showed a 23-fold reduction in IC50, but in those with 10% BHK cd^+ cells, the reduction was only 6-fold. These variations were most likely caused by the higher cd activity seen in L929 cd^+ cells (Table 5.2.). Increasing the percentage of cd^+ cells in the culture caused a progressive reduction of the IC50 and cultures containing only cd^+ cells showed a therapeutic index of >100 (figure 5.2.; Table 5.2.).

The above analyses have demonstrated that expression of the tp gene or the cd gene can sensitise these cell lines to the appropriate prodrug and in both enzyme / prodrug systems, toxicity can spread to untransfected cells, irrespective of whether or not there are functional gap junctions. However, bystander killing in these systems was <10% as effective when

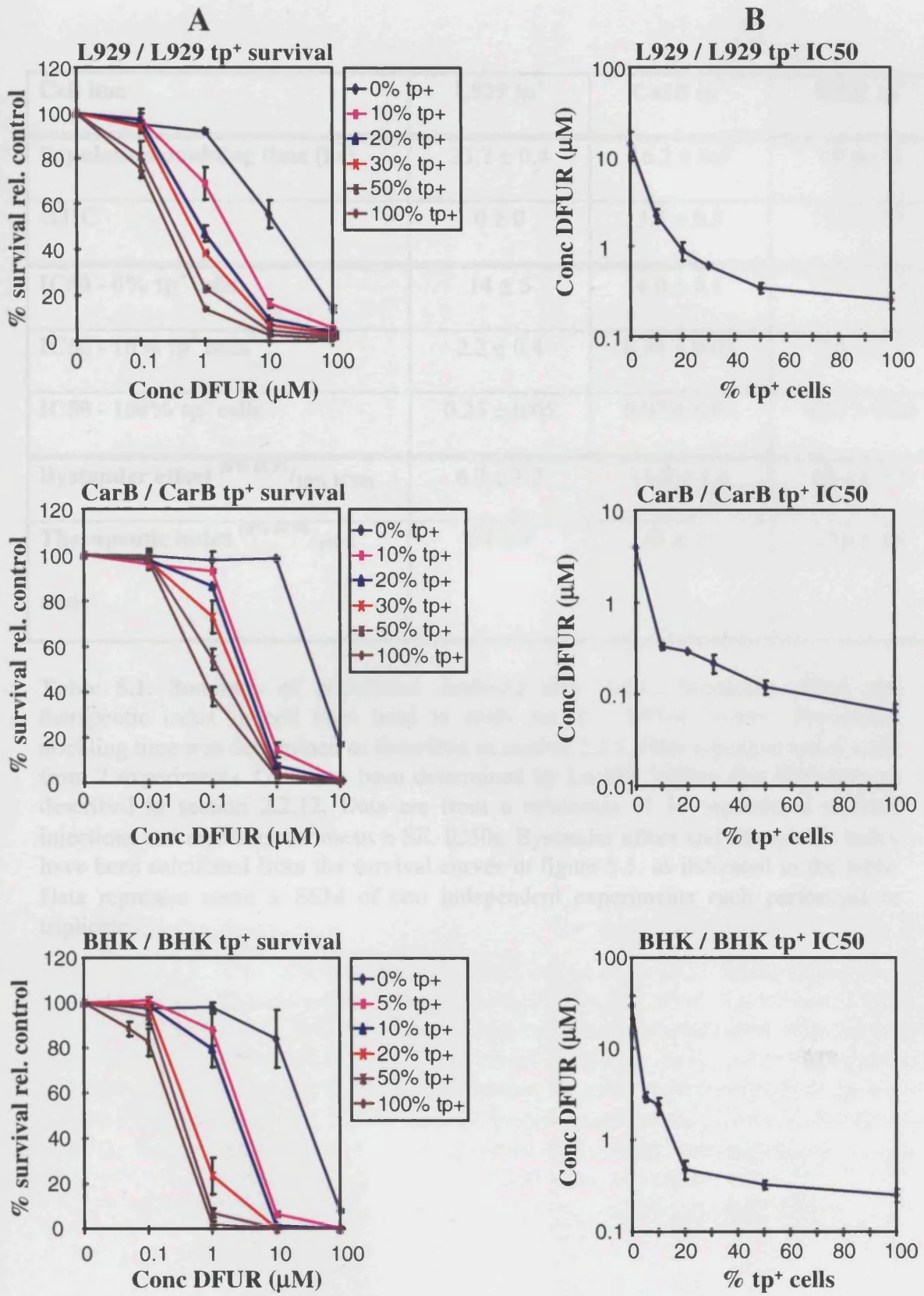


Figure 5.1. Survival and IC50 of mixed populations of tp^+ and tp^- cells. **(A)** Varying mixtures of tp^+ and tp^- cells were seeded in 24 well tissue culture plates (L929, 1 x 10^5; CarB 1.5 x 10^5; BHK, 5 x 10^4 cells / well). 24 hr later, cells were treated with DFUR for 2 days. After this time cells from each well were released with trypsin. Aliquots of the suspension were transferred to 96 well plates containing media and DFUR, as described in section 2.2.7. Survival was determined using the MTT assay. By calculation from each of the survival curves in **(A)**, the IC50, with respect to DFUR, for each of the cell mixtures was calculated **(B)**. Each data point represents the mean +/_ SEM (bars) of two independent experiments, each performed in triplicate. L929 cells lack GJIC whereas CarB and BHK cells communicate. All show a bystander effect.

Cell line	L929 tp^+	CarB tp^+	BHK tp^+
Population doubling time (hr)	23.7 ± 0.4	16.2 ± 0.4	10.9 ± 0
GJIC	0 ± 0	3.4 ± 0.5	11.9 ± 1.0
IC50 - 0% tp^+ cells	14 ± 5	4.0 ± 0.1	29.5 ± 8.5
IC50 - 10% tp^+ cells	2.2 ± 0.4	0.34 ± 0.03	2.4 ± 0.5
IC50 - 100% tp^+ cells	0.25 ± 0.05	0.07 ± 0.01	0.25 ± 0.04
Bystander effect $^{(0\%\ IC50}/_{10\%\ IC50)}$	6.2 ± 1.2	11.9 ± 1.1	12.1 ± 1.0
Therapeutic index $^{(0\%\ IC50}/_{100\%\ IC50)}$	54 ± 9	63 ± 13	116 ± 16

Table 5.1. Summary of population doubling time, GJIC, bystander effect and therapeutic index in cell lines used to study the tp / DFUR system. Population doubling time was determined as described in section 2.2.5. Data represent mean ± SE from 2 experiments. GJIC has been determined by Lucifer Yellow dye injections as described in section 2.2.12. Data are from a minimum of 10 separate, 2 minute, injections and represent the mean ± SE. IC50s. Bystander effect and therapeutic index have been calculated from the survival curves in figure 5.1. as indicated in the table. Data represent mean ± SEM of two independent experiments each performed in triplicate.

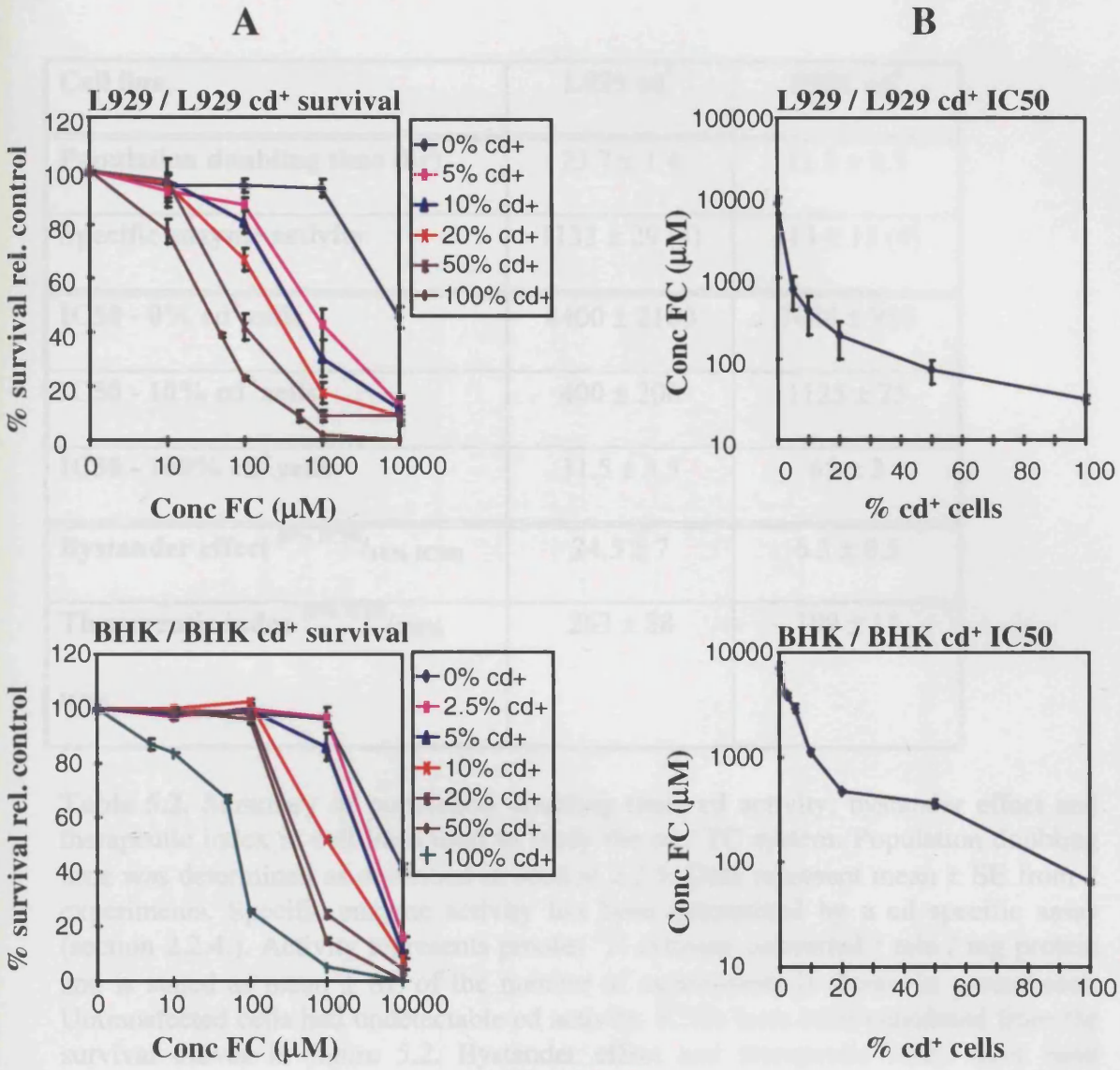


Figure 5.2. Survival and IC50 of mixed populations of cd^+ and cd^- cells. **(A)** Varying mixtures of cd^+ and cd^- cells were seeded in 24 well tissue culture plates (L929, 1 x 10^5; BHK, 5 x 10^4 cells / well). 24 hr later, cells were treated with FC for 2 days. After this time cells from each well were released with trypsin. Aliquots of the suspension were transferred to 96 well plates containing media and FC, as described in section 2.2.7. Survival was determined using the MTT assay. By calculation from each of the survival curves in **(A)**, the IC50, with respect to FC, for each of the cell mixtures was calculated **(B)**. Each data point represents the mean +/_ SEM (bars) of two independent experiments, each performed in triplicate. L929 cells lack GJIC whereas BHK cells communicate. Both show a bystander effect.

Cell line	L929 cd^+	BHK cd^+
Population doubling time (hr)	23.7 ± 1.4	11.5 ± 0.5
Specific enzyme activity	1133 ± 29 (4)	413 ± 15 (4)
IC50 - 0% cd^+ cells	8400 ± 2100	7050 ± 950
IC50 - 10% cd^+ cells	400 ± 200	1125 ± 75
IC50 - 100% cd^+ cells	31.5 ± 3.5	65 ± 3
Bystander effect $^{(0\%\ IC50}/_{10\%\ IC50)}$	24.5 ± 7	6.3 ± 0.5
Therapeutic index $^{(0\%\ IC50}/_{100\%\ IC50)}$	263 ± 38	109 ± 11

Table 5.2. Summary of population doubling time, cd activity, bystander effect and therapeutic index in cell lines used to study the cd / FC system. Population doubling time was determined as described in section 2.2.5. Data represent mean ± SE from 2 experiments. Specific enzyme activity has been determined by a cd specific assay (section 2.2.4.). Activity represents pmoles ^3H cytosine converted / min / mg protein and is stated as mean ± SE of the number of experiments is shown in parentheses. Untransfected cells had undetectable cd activity. IC50s have been calculated from the survival curves in figure 5.2. Bystander effect and therapeutic index have been determined from these values, as indicated in the table. Data represent mean ± SEM of two independent experiments each performed in triplicate.

Suicide gene	**tk**		**tp**		**cd**	
Cell line	**Byst. Eff.**	**Ther. Ind.**	**Byst. Eff.**	**Ther. Ind.**	**Byst. Eff.**	**Ther. Ind.**
L929	1.04	3715	6.2	54	24.5	263
MCF-7	1.06	2242	-	-	-	-
CarB	351	1492	11.9	63	-	-
BHK	292	3530	12.1	116	6.3	109
CarB-E	282	1134	-	-	-	-
B9	273	828	-	-	-	-

Table 5.3. Summary of bystander effects and therapeutic indicies in cell lines used to study the tk / GCV, tp / DFUR and cd / FC systems. Data have been collected from tables 3.1. (tk), 5.1. (tp) and 5.2. (cd). Bystander effects (Byst. Eff.) were calculated as $^{\text{IC50 0\% gene+}}/_{\text{IC50 10\% gene+}}$ and therapeutic indicies (Ther. Ind.) as $^{\text{IC50 0\% gene+}}/_{\text{IC50 100\% gene+}}$. All values represent the mean of two experiments each performed in triplicate.

compared to the tk / GCV system, in coupled cells, and this is considered further in section 5.5.

5.3. Transfer of toxicity via the medium in tp / DFUR and cd / FC systems.

Previous reports have demonstrated the release of FU to the medium when cd^+ cells are treated with FC (Huber et al, 1995). Results in the last section showed that transfer of toxicity to untransfected cells in the tp / DFUR and cd / FC systems was independent of gap junctions, and although it is likely that this was via the medium, it is important to investigate this suggestion in order to compare these systems with the tk / GCV combination.

In medium transfer experiments, cultures of tp^+ and cd^+ cells were treated, for 2 days, with DFUR or FC, respectively, at concentrations known to produce a bystander effect. The medium was then transferred to untransfected cells and survival was measured in the same way that has been described previously for the tk / GCV system (section 2.2.9.; section 3.3.3.).

Medium transferred from DFUR treated tp^+ cells reduced survival of tp^- cells by >90% in all the cell lines tested when compared to controls, where tp^+ cells were cultured without the prodrug, or where tp^- cells were treated with the prodrug (figure 5.3.A.). Similar results were seen in parallel experiments with the cd / FC system (figure 5.4.A.).

Support for the above results came from the observation that separation of tp^+ and tp^- cells (figure 5.3.B.) or of cd^+ and cd^- cells (figure 5.4.B.) with an Anopore filter membrane did not prevent spread of toxicity to untransfected cells.

It can be concluded that toxicity was transferred via the medium and that cell contact hence gap junctions, are not required for a bystander effect in the tp / DFUR or cd / FC

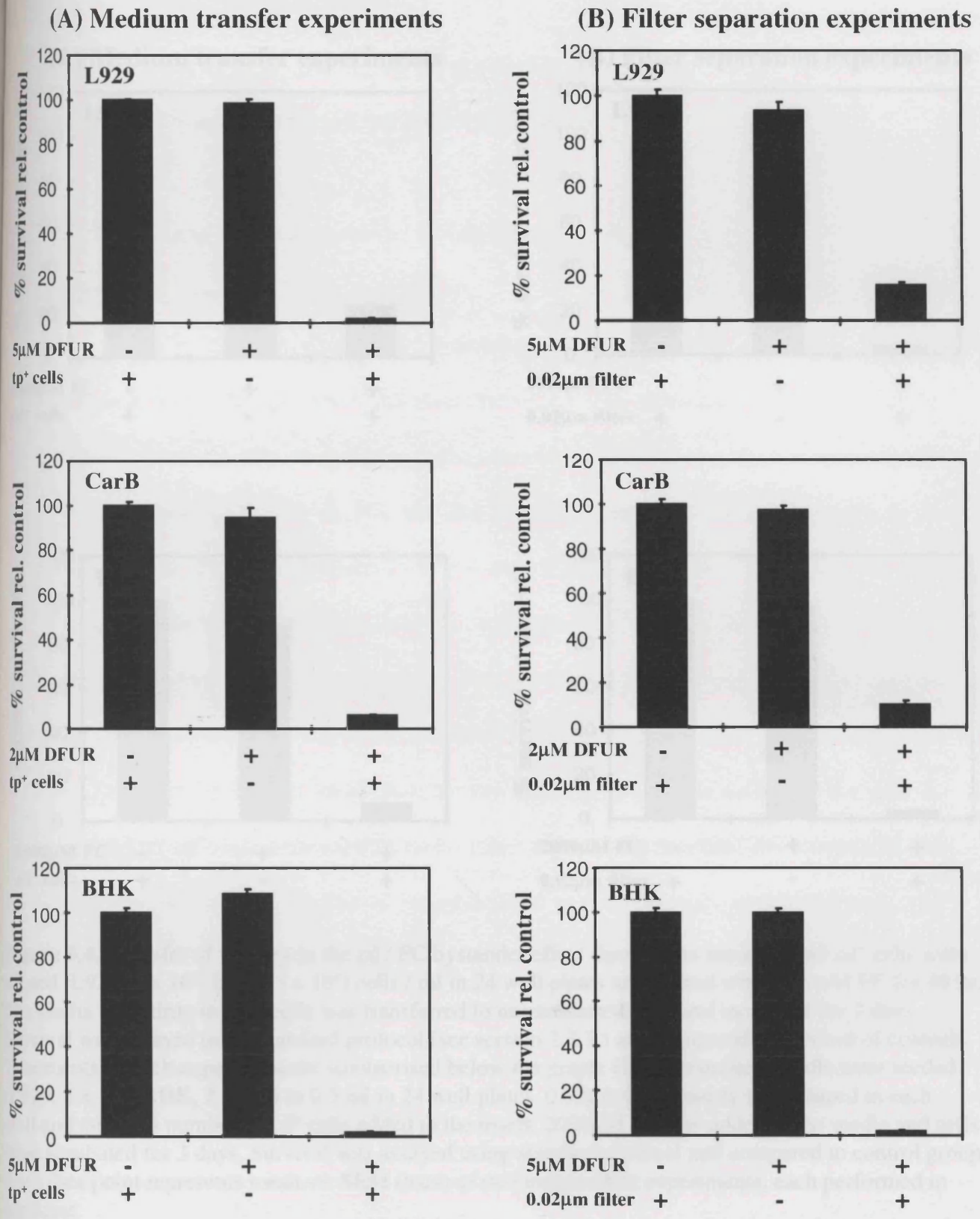


Figure 5.3. Transfer of toxicity in the tp/DFUR bystander effect through the medium. **(A)** tp^+ cells were seeded (L929 and CarB, 1 x 10^5; BHK, 5 x 10^4) cells / ml in 24 well plates and treated with DFUR for 48 hr. The media from drug treated cells was transferred to untransfected cells and incubated for 2 days. Survival was assayed using standard protocol (see section 2.2.7.) and compared to survival of controls. Treatments in each experiment are summarised below the graph. **(B)** Untransfected cells were seeded (L929, 5 x 10^4; CarB, 7 x 10^4;BHK, 2 x 10^4) in 0.5 ml in 24 well plates. 0.02μm filter inserts were placed in each well and an equal number of tp^+ cells added to the insert. DFUR was added to the media and cells were incubated for 3 days. Survival was assayed using standard protocol and compared to control groups. Each data point represents mean +/- SEM (bars) of two independent experiments, each performed in triplicate.

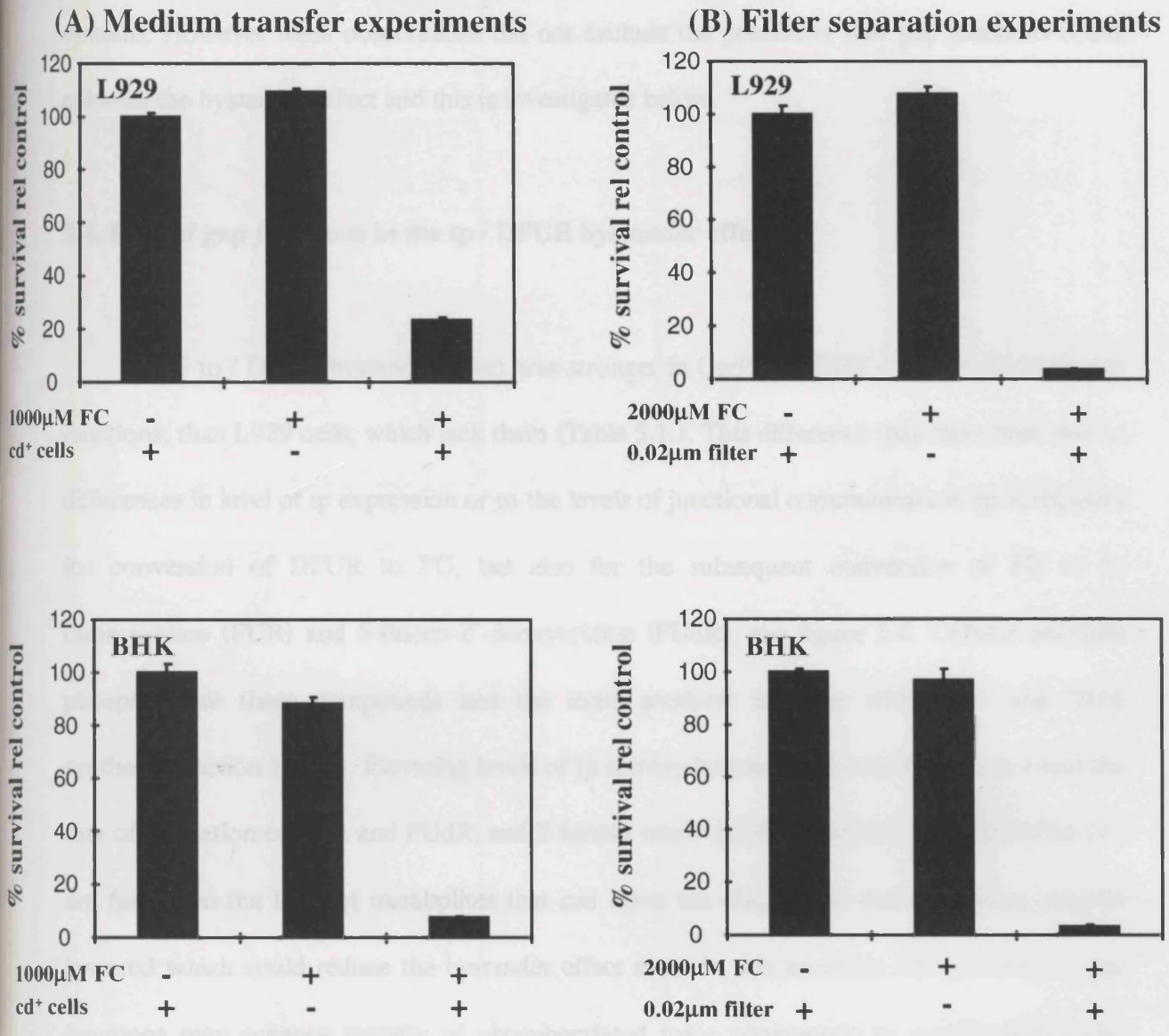


Figure 5.4. Transfer of toxicity in the cd / FC bystander effect through the medium. **(A)** cd^+ cells were seeded (L929, 1 x 10^5; BHK, 5 x 10^4) cells / ml in 24 well plates and treated with 1000μM FC for 48 hr. The media from drug treated cells was transferred to untransfected cells and incubated for 2 days. Survival was assayed using standard protocol (see section 2.2.7.) and compared to survival of controls. Treatments in each experiment are summarised below the graph. **(B)** Untransfected cells were seeded (L929, 5 x 10^4; BHK, 2 x 10^4) in 0.5 ml in 24 well plates. 0.02μm filter inserts were placed in each well and an equal number of cd^+ cells added to the insert. 2000μM FC was added to the media and cells were incubated for 3 days. Survival was assayed using standard protocol and compared to control groups. Each data point represents mean +/- SEM (bars) of two independent experiments, each performed in triplicate.

systems. However these observations did not exclude the possibility that gap junctions could enhance the bystander effect and this is investigated below.

5.4. Role of gap junctions in the tp / DFUR bystander effect.

The tp / DFUR bystander effect was stronger in CarB and BHK cells, which have gap junctions, than L929 cells, which lack them (Table 5.1.). This difference may have been due to differences in level of tp expression or to the levels of junctional communication. tp is required for conversion of DFUR to FU, but also for the subsequent conversion of FU to 5-fluorouridine (FUR) and 5-fluoro-2'-deoxyuridine (FUdR), see figure 1.2. Cellular enzymes phosphorylate these compounds and the toxic products interfere with RNA and DNA synthesis (section 1.10.2). Elevating levels of tp activity by transfection is likely to increase the rate of formation of FUR and FUdR, and if further reactions (which result in phosphorylation) are fast, then the level of metabolites that can leave the cell, across the membrane, may be lowered which could reduce the bystander effect seen. In this situation, the presence of gap junctions may enhance transfer of phosphorylated toxic compounds to neighbouring cells, hence enhance the bystander effect.

To test this hypothesis, L929 and BHK cells were used to compare the tk / GCV and tp / DFUR systems. The tk / GCV system acted as a control since gap junctions are required for transfer of toxicity. Mixed cultures of 10% tk^+ or 10% tp^+ cells and 90% wild type cells were seeded at high or low density (50 fold difference) and survival of cultures in response to increasing concentrations of GCV or DFUR, respectively, was measured (see section 2.2.7.; figure 5.5).

At high or low density, the survival of mixed cultures containing L929 tk^+ cells, was not significantly different, which was expected since these cells are not coupled (figure 5.5.A.).

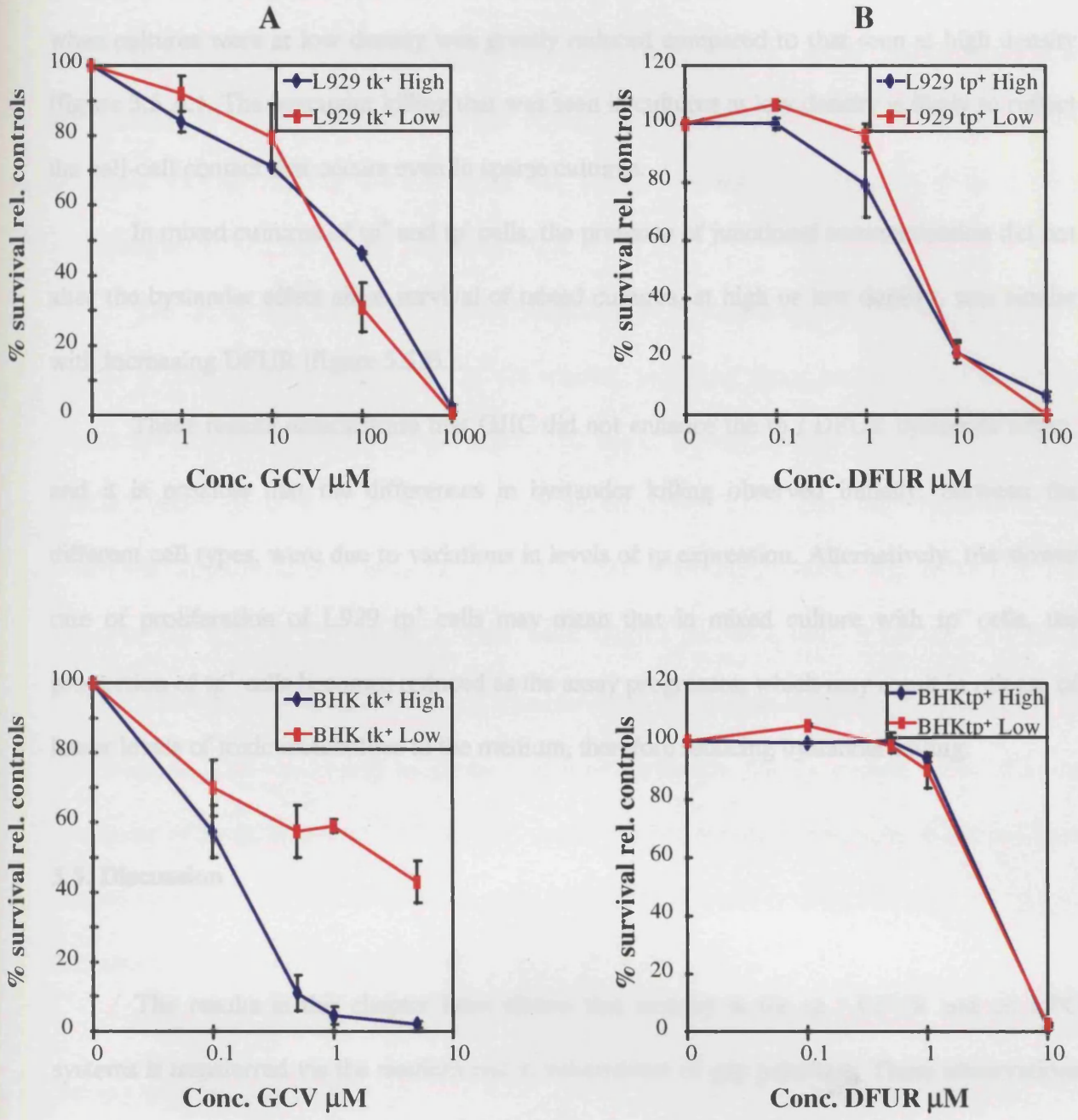


Figure 5.5. Gap junctions do not improve the tp / DFUR bystander effect. 10% tk^+ **(A)** or 10% tp^+ **(B)** cells were mixed with 90% wild type cells to a high (L929, 1 x 10^5; BHK 5 x 10^4 cells / well) or low (L929, 2 x 10^3; BHK 1 x 10^3 cells / well) density in 24 well tissue culture plates. 24 hr later cells were treated with GCV or DFUR, as indicated, for 2 days. After this time cells from each well were released with trypsin. Aliquots of the suspension were transferred to 96 well plates containing media with GCV or DFUR, as described in section 2.2.7. Survival was determined using the MTT assay and compared to untreated controls. Each data point represents the mean +/_ SEM (bars) of two independent experiments, each performed in triplicate. L929 cells lack GJIC, whereas BHK cells communicate.

This contrasted to mixed cultures containing BHK tk^+ cells, where the bystander effect seen when cultures were at low density was greatly reduced compared to that seen at high density (figure 5.5.A.). The bystander killing that was seen in cultures at low density is likely to reflect the cell-cell contact that occurs even in sparse cultures.

In mixed cultures of tp^+ and tp^- cells, the presence of junctional communication did not alter the bystander effect since survival of mixed cultures, at high or low density, was similar with increasing DFUR (figure 5.5.B.).

These results demonstrate that GJIC did not enhance the tp / DFUR bystander effect, and it is possible that the differences in bystander killing observed initially, between the different cell types, were due to variations in levels of tp expression. Alternatively, the slower rate of proliferation of L929 tp^+ cells may mean that in mixed culture with tp^- cells, the proportion of tp^+ cells becomes reduced as the assay progresses, which may result in release of lower levels of toxic metabolites to the medium, therefore reducing bystander killing.

5.5. Discussion

The results in this chapter have shown that toxicity in the tp / DFUR and cd / FC systems is transferred via the medium and is independent of gap junctions. These observations are consistent with previous reports using the cd / FC system (Hirschowitz et al, 1995; Haberkorn et al, 1996b) and with a recent report, investigating the tp / DFUR system (Kato et al, 1997).

The data here have shown that in cultures containing 10% tp^+ cells the IC50 is reduced by 5- to 10-fold compared with wild type cells, while in those with 100% tp^+ cells, the IC50 is reduced by 50- to 100-fold. Similar reductions have been observed in other cell lines transfected with the tp gene (Patterson et al, 1995; Kato et al, 1997). The authors of these

reports demonstrated that transfection with the tp gene increased activity by up to 100-fold compared to parental cells, however, the data presented here show that similar increases in tk enzyme activity lead to substantially larger increases in sensitivity to GCV. For example, BHK tk clones, with activities of >40 fold higher than parental cells, have a bystander effect of >100 and therapeutic index of >1000 (section 3.3.2.).

The apparently consistent, limited enhancement of sensitivity seen in the tp / DFUR system is disappointing because all of the studies, including those here, have achieved tp expression from a strong (CMV) promoter. The use of a tissue specific promoter is likely to produce lower levels of tp expression, which can be expected to reduce the bystander effect. There also appears to be a large variation in sensitivity of untransfected cells to DFUR. For example, the IC50 of CarB cells is 4μM while that of human epidermal carcinoma cells is 250μM (Haraguchi et al, 1993). Even if transfection of the tp gene sensitised the latter cells by 100-fold, the IC50 would only be similar to that of CarB cells. Such a marked variation in the sensitivity of parental cells to GCV and FC appears not to occur, for example, in the cell lines studied here, there is <3-fold variation and a survey of the literature suggests this may be true for other cell lines of different origins.

These observations suggest that the use of the tp / DFUR system for cancer gene therapy may be restricted to tumours which have high levels of endogenous tp activity, for example, those of the breast, cervix and stomach (Yamamoto et al, 1996), where further increasing the level of tp expression may be beneficial.

The studies here, using the cd / FC system, have demonstrated a relatively weak bystander effect and a therapeutic index of <300, which contrasts to numerous previous reports where stronger bystander effects and higher therapeutic indices were seen (Huber et al, 1995; Trinh et al, 1995; Hoganson et al, 1996; Li et al, 1997; Kanai et al, 1997). In some cases, these differences may be accounted for by variations in the level of cd activity. For

example, expression of cd was 50- to 100-fold higher in hepatocellular carcinoma or breast cancer cell lines, after adenoviral delivery of the cd gene (Li et al, 1997; Kanai et al, 1997), compared with the cell lines studied here. However, the level of enzyme activity does not always explain differences in sensitivity, since transfection of a lung adenocarcinoma cell line with the cd gene (Hoganson et al, 1996) produced a similar level of expression, but a stronger bystander effect, compared to those seen in L929 cd^+ and BHK cd^+. It is possible that FU sensitivities of these cell lines are different.

These observations highlight the importance of selecting an appropriate regime for treatment of different tumours. In the cell lines studied here, the tk / GCV system is more effective than the cd / FC system, while the latter strategy is more effective in other cell lines. In some tumours, the use of the tp / DFUR combination may be more effective.

Chapter 6. Results - Combination of enzyme / prodrug systems

6.1. Introduction.

Clinical trials involving the intratumoural implantation of retroviral vector producer cells, in patients with glioblastoma multiforme, have demonstrated low transduction efficiencies (<0.2% of tumour cells) with the tk gene, and subsequent GCV treatment resulted in a reduction in tumour size in only a fraction of patients (Culver et al, 1996; Izquierdo et al, 1996). For gene therapy to play a role in the treatment of cancer, it will be important to increase the transduction efficiency of tumour cells and to enhance the bystander effect.

Bystander killing in tk / GCV system depends on gap junctions and the toxic metabolite that is transferred inhibits DNA synthesis, while in tp / DFUR or cd / FC strategies, toxicity passes through the medium and interferes with both DNA and RNA synthesis. It is possible that combining these systems, that have bystander effects operating by different pathways or that kill cells by different mechanisms, may enhance untransfected cell killing. The experiments in this chapter investigate the effect of combining the tk / GCV system with either the tp / DFUR system or the cd / FC system.

6.2. Combining the tk / GCV and tp / DFUR systems.

L929 cells do not form gap junctions and were excluded from these studies, since both suicide gene systems must have a bystander effect. Double transfection of CarB or BHK cells with the tk gene, and subsequently the tp gene, has been described previously in sections 3.2.2. and 5.2.

Mixed cultures of 10% tk^+-tp^+ (CarB or BHK) with 90% wild type cells were treated with GCV and / or DFUR for two days and survival was measured in the same way that has been described previously (section 3.3.1.; section 5.2.2.; section 2.2.7.). At high or low concentrations of prodrug there was no significant difference in survival between single or combined treatments, but at intermediate doses, an increased bystander effect was seen with the combined treatment (figure 6.1.). For example, 1μM GCV reduced survival of CarB mixed cultures (10% tk^+-tp^+ : 90% wild type) to 28% and 1μM DFUR reduced survival to 33% but when combined, survival was reduced to 13%, compared to untreated controls (figure 6.1.).

There was a similar trend in 10% BHK tk^+-tp^+ : 90% BHK mixed cultures. No significant difference in survival was seen between treatment groups at high or low prodrug concentrations, but while separately, 0.1μM GCV or 1μM DFUR did not cause a significant level of cell death in the mixed cultures, the combined treatment killed 50% of cells (figure 6.1.).

The data from these cell lines show that combining the tk / GCV and tp / DFUR strategies can, in the right circumstances, lead to an enhanced bystander effect compared to treatment with only one prodrug.

Transfer of toxicity is via gap junctions in the tk / GCV system and via the medium in the tp / DFUR system. It is possible that high, local concentrations of GCV-phosphates are transferred to neighbouring cells, but FU in the medium may spread more uniformly to both near and distant cells. GCV may therefore kill the nearby cells more effectively, while the distant cells may be killed by DFUR. Alternatively, when one system is used alone, the concentration of toxic metabolites in a proportion of the cells may be too low to cause cell death but, by combining treatments, the level of toxicity may be raised above a threshold, killing additional cells. If this latter hypothesis is correct, it would be important to co-ordinate prodrug delivery so the peaks of toxic metabolite production coincide.

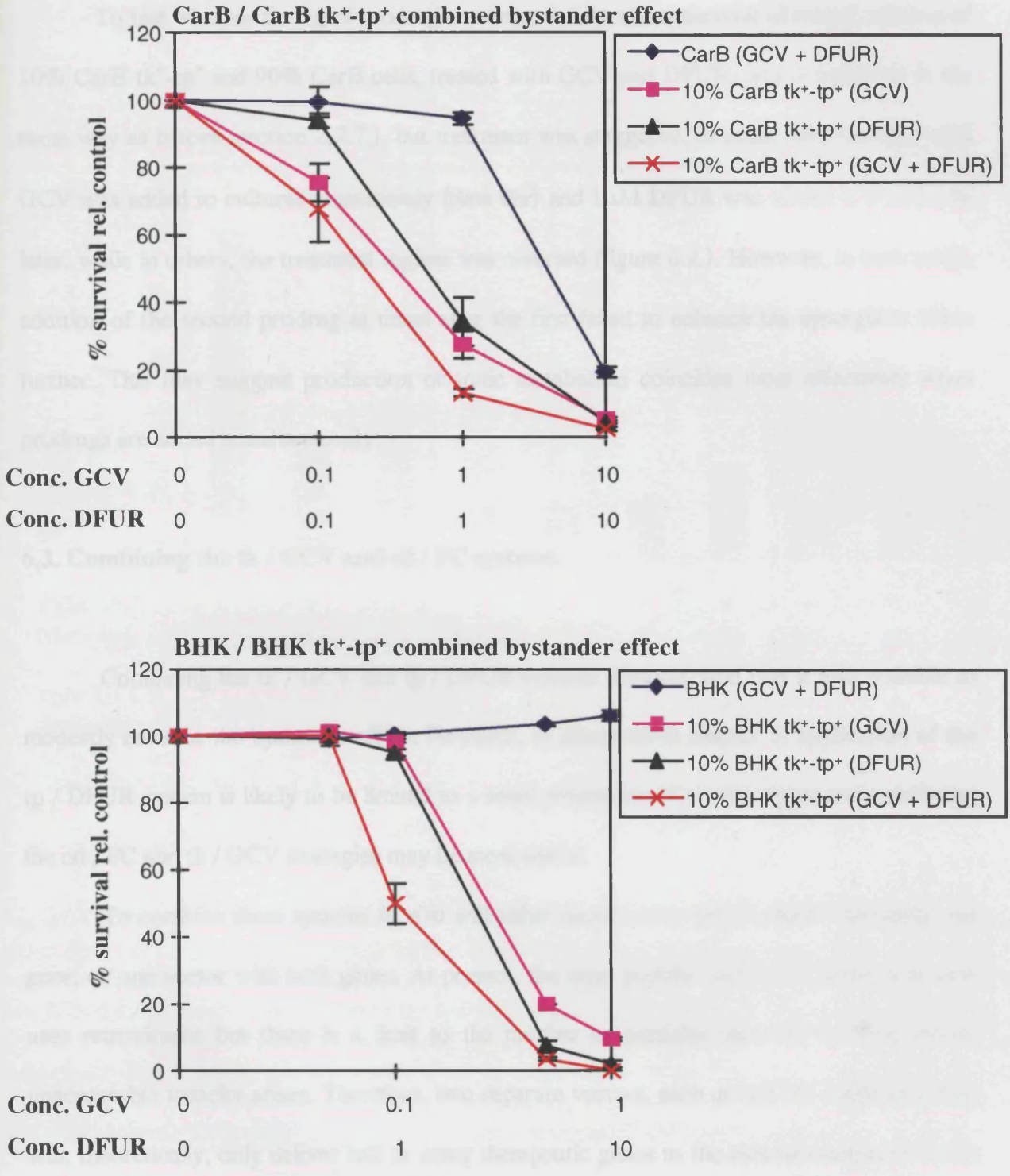


Figure 6.1. Combining the tk / GCV and tp / DFUR enzyme / prodrug systems results in an improved bystander effect at intermediate prodrug concentrations. 10% CarB tk^+-tp^+ or 10% BHK tk^+-tp^+ double transfected cells were mixed with 90% parental cells in 24 well plates (CarB 1.5 x 10^5; BHK 5 x 10^4 cells / ml). 24 hr later increasing concentrations of either GCV, DFUR or both were added to the media, as indicated in the graphs above. Survival was assayed using standard protocol (see section 2.2.7.) and expressed as percentage growth inhibition of untreated cells. All data represent mean +/- SEM (bars) of two independent experiments, each performed in triplicate with the exception of CarB tk^+-tp^+ cultures treated with 1µM GCV and / or 1µM DFUR where data are mean +/- SEM of three independent experiments, each performed in triplicate.

To test whether timing of prodrug treatment is important, survival of mixed cultures of 10% CarB tk^+-tp^+ and 90% CarB cells, treated with GCV and DFUR, was determined in the same way as before (section 2.2.7.), but treatment was staggered. In some experiments, 1μM GCV was added to cultures immediately (time 0hr) and 1μM DFUR was added 4, 8 or 24 hr later, while in others, the treatment regime was reversed (figure 6.2.). However, in both cases, addition of the second prodrug at times after the first failed to enhance the synergistic effect further. This may suggest production of toxic metabolites coincides most effectively when prodrugs are added simultaneously.

6.3. Combining the tk / GCV and cd / FC systems.

Combining the tk / GCV and tp / DFUR systems demonstrated that it was possible to modestly enhance the bystander effect. However, as discussed in chapter 5, application of the tp / DFUR system is likely to be limited to a small proportion of tumour types and combining the cd / FC and tk / GCV strategies may be more useful.

To combine these systems *in vivo* will either require two vectors, each expressing one gene, or one vector with both genes. At present, the most popular method of delivery *in vivo* uses retroviruses but there is a limit to the number or particles that can be used before unacceptable toxicity arises. Therefore, two separate vectors, each at half the maximum dose will, theoretically, only deliver half as many therapeutic genes to the tumour compared to the one vector system. There are also other issues to be considered before designing a suitable strategy for combined gene delivery.

Separate expression of two foreign genes from their own promoters in retroviral vectors has been associated with interference. For example, in a recombinant retrovirus carrying neo and tk genes, expression of the tk gene was repressed when the neo gene was

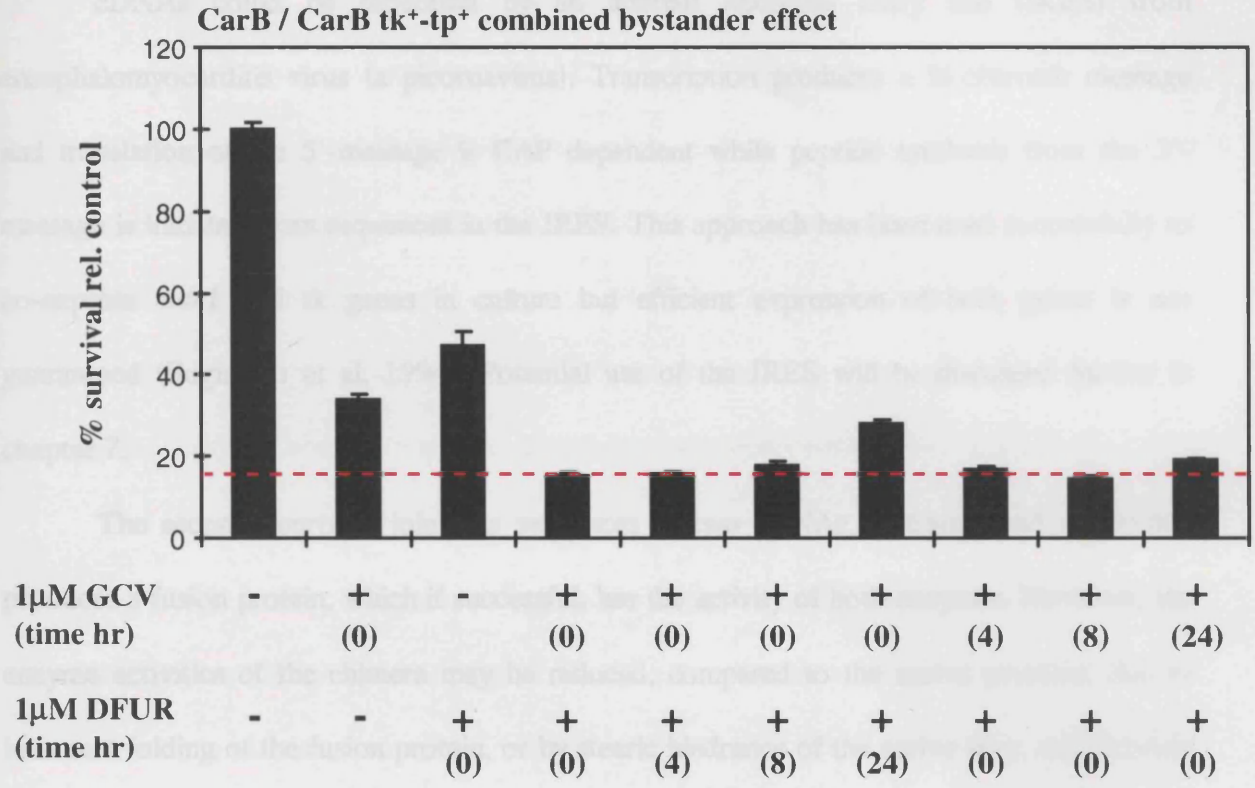


Figure 6.2. Varying time of prodrug application in the tk / GCV and tp / DFUR combined bystander effect does not result in improved cell killing. 10% CarB tk^+-tp^+ double transfected cells were mixed with 90% CarB cells in 24 well plates (1.5 x 10^5 cells / well). After 24 hr 1µM GCV or 1µM DFUR was added to the medium and at the times specified above (parentheses, hr) the second prodrug (1µM DFUR or 1µM GCV) was added. 48 hr from the first prodrug treatment cells were transferred to 96 well plates. Survival was assayed as described in section 2.2.7. and compared to untreated controls. The dotted line indicates the extent of cell death when both prodrugs are added simultaneously at time 0 hr. Data represent the mean +/- SE (bars) of one experiment performed in triplicate.

expressed, and visa versa (Emerman and Temin, 1984). Ideally one promoter should drive expression of two genes.

cDNAs could be separated by an internal ribosome entry site (IRES) from encephalomyocarditis virus (a picornavirus). Transcription produces a bi-cistronic message and translation of the 5'-message is CAP dependent while peptide synthesis from the 3'-message is initiated from sequences in the IRES. This approach has been used successfully to co-express *mdr1* and tk genes in culture but efficient expression of both genes is not guaranteed (Sugimoto et al, 1994). Potential use of the IRES will be discussed further in chapter 7.

The second approach joins the sequences of two cDNAs, in frame, and expression produces a fusion protein, which if successful, has the activity of both enzymes. However, the enzyme activities of the chimera may be reduced, compared to the native proteins, due to incorrect folding of the fusion protein, or by stearic hindrance of the active sites, which could decrease the value of such an approach. Currently the folding patterns of peptides cannot be predicted, and until tested, the feasibility of fusion proteins cannot be judged.

Another consideration is whether a longer transcript can be delivered efficiently by vectors. Retroviral delivery of a GCSF-IRES-tk/neo construct resulted in production of the cytokine, granulocyte colony stimulating factor, and also a tk-neo fusion protein (Veelken et al, 1996). This fusion chimera caused cellular sensitivity to GCV and resistance to G418, but the number of G418 resistant colonies was reduced by 50% compared to a GCSF-IRES-neo construct. This may have been due to incorrect folding of the fusion protein or an excessive transcript length.

Despite the potential problems, expression of some fusion proteins has been successful. For example, fibrosarcoma cells expressing a tk-neo construct (see above) had both enzyme activities (Veelken et al, 1996) and when hygromycin phosphotransferase was fused to the N-

terminus of tk (hyg-tk), NIH3T3 cells showed resistance to hygromycin and sensitivity to GCV (Lupton et al, 1991). Alternatively, when the cd gene was fused to the cDNA of the cellular marker, CD44, (CD44-cd) and used to transform E. coli cd$^-$ mutants, deamination of cytosine was as efficient as that of wild type E. coli strains (Asman et al, 1995). This suggests that fusion of proteins to the N- or C-terminus of tk, and the N-terminus of cd, did not cause stearic hindrance, at least in some cases. However, the pattern of folding of each fusion protein will be different and, as this cannot be predicted, tk-cd or cd-tk fusion chimeras were constructed. The strategy used for the cd-tk fusion is shown in figure 6.3. and by the same process, but using different primers, the tk-cd fusion was generated (section 2.5.5.).

The suicide genes in both constructs were separated by an XbaI restriction site, which is 6 base pairs in length, hence fuses the cDNAs in frame. The XbaI sequence translates to serine and lysine residues. Transcription of the chimeras was driven by the CMV promoter and the Kozak sequence was added to enhance translation (Kozak, 1986). The pCDNA3.1$^-$ expression plasmid also has a neo selection marker.

Transient transfections (calcium phosphate; see section 2.2.3.) with both constructs were performed to determine whether expression of these fusion proteins produced sensitivity to either, or both, prodrugs. Initial screening showed that killing of cells was achieved with 10μM GCV or 1000μM FC, but less cell death was seen with FC treatment in cells transfected with tkcd than with those transfected with cdtk.

Subsequent stable transfections with the tkcd construct failed to produce clones that were sensitive to either prodrug. It is possible that if only a small proportion of the tkcd fusion proteins were correctly folded, or if the activity of each molecule was low, then killing may be seen in transient, but not in stable, transfections. Transient transfection of genes results in more cells expressing higher levels of the construct than in stable transfections.

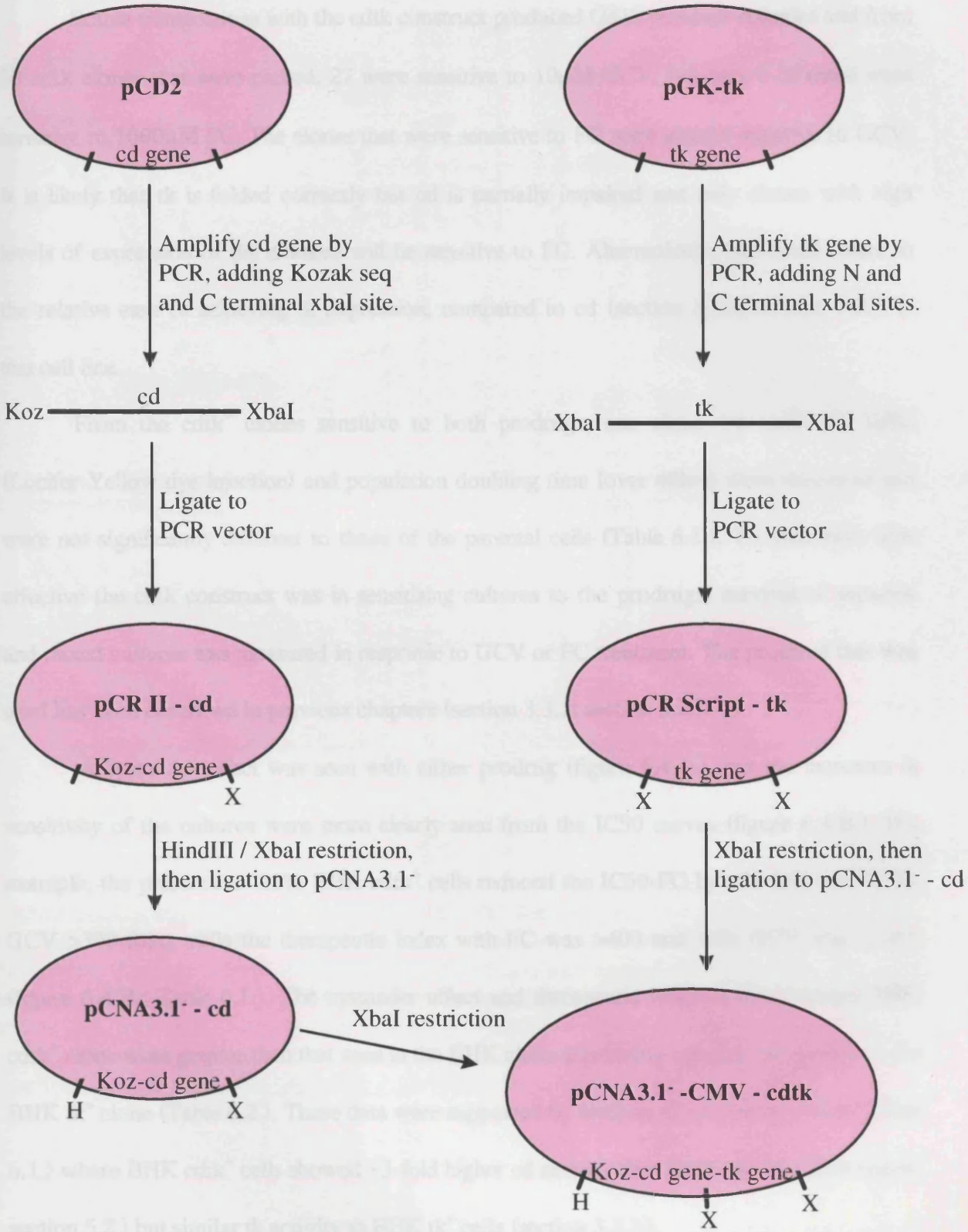


Figure 6.3. Cloning strategy for producing the cytosine deaminase-thymidne kinase fusion chimera. The cd gene was amplified by PCR using primers with the sequence GCC GCC ACC ATG TCG AAT AAC GCT TTA CAA and GTG GTG TCT AGA ACG TTT GTA ATC GAT GGC TTC. The PCR product was cloned into the Invitrogen cloning vector (pCR II) and then subcloned to the expression vector pCDNA3.1-. The tk gene was amplified by PCR using primers with the sequence GAG GAG TCT AGA ATG GCT TCG TAC CCC and GTG GTG TCT AGA TCA GTT AGC CTC CCC CAT. The product was cloned into the Stratagene pCR Script vector. Subcloning tk into pCDNA3.1- - cd generated a cdtk chimera, linked by an XbaI sequence and driven by a CMV promoter.

Stable transfections with the cdtk construct produced G418 resistant colonies and from 30 cdtk clones that were picked, 27 were sensitive to 10μM GCV, but only 6 of these were sensitive to 1000μM FC. The clones that were sensitive to FC were always sensitive to GCV. It is likely that tk is folded correctly but cd is partially impaired and only clones with high levels of expression of the chimera will be sensitive to FC. Alternatively, this could relate to the relative ease of achieving tk expression, compared to cd (section 3.2.2; section 5.2.), in this cell line.

From the cdtk$^+$ clones sensitive to both prodrugs, one clone was selected. GJIC (Lucifer Yellow dye injection) and population doubling time (over 48hrs) were measured and were not significantly different to those of the parental cells (Table 6.1.). To determine how effective the cdtk construct was in sensitising cultures to the prodrugs, survival of separate and mixed cultures was measured in response to GCV or FC treatment. The protocol that was used has been described in previous chapters (section 3.3.1; section 5.2.).

A bystander effect was seen with either prodrug (figure 6.4.A.), but the increases in sensitivity of the cultures were more clearly seen from the IC50 curves (figure 6.4.B.). For example, the presence of 10% BHK cdtk$^+$ cells reduced the IC50-FC by ~23 fold and IC50-GCV >300 fold, while the therapeutic index with FC was >400 and with GCV was >2000 (figure 6.4.B.; Table 6.1.). The bystander effect and therapeutic index in this selected BHK cdtk$^+$ clone were greater than that seen in the BHK clone expressing only cd, but similar to the BHK tk$^+$ clone (Table 6.2.). These data were supported by analysis of enzyme activities (Table 6.1.) where BHK cdtk$^+$ cells showed ~3-fold higher cd activity than BHK cd$^+$ cells (see below; section 5.2.) but similar tk activity to BHK tk$^+$ cells (section 3.3.1.).

To test if the bystander effects observed with cells expressing the chimeric protein were similar to those seen with cells expressing the enzymes separately, BHK cd$^+$ cells were transfected with a plasmid carrying the tk and hyg genes. Hygromycin resistant clones were

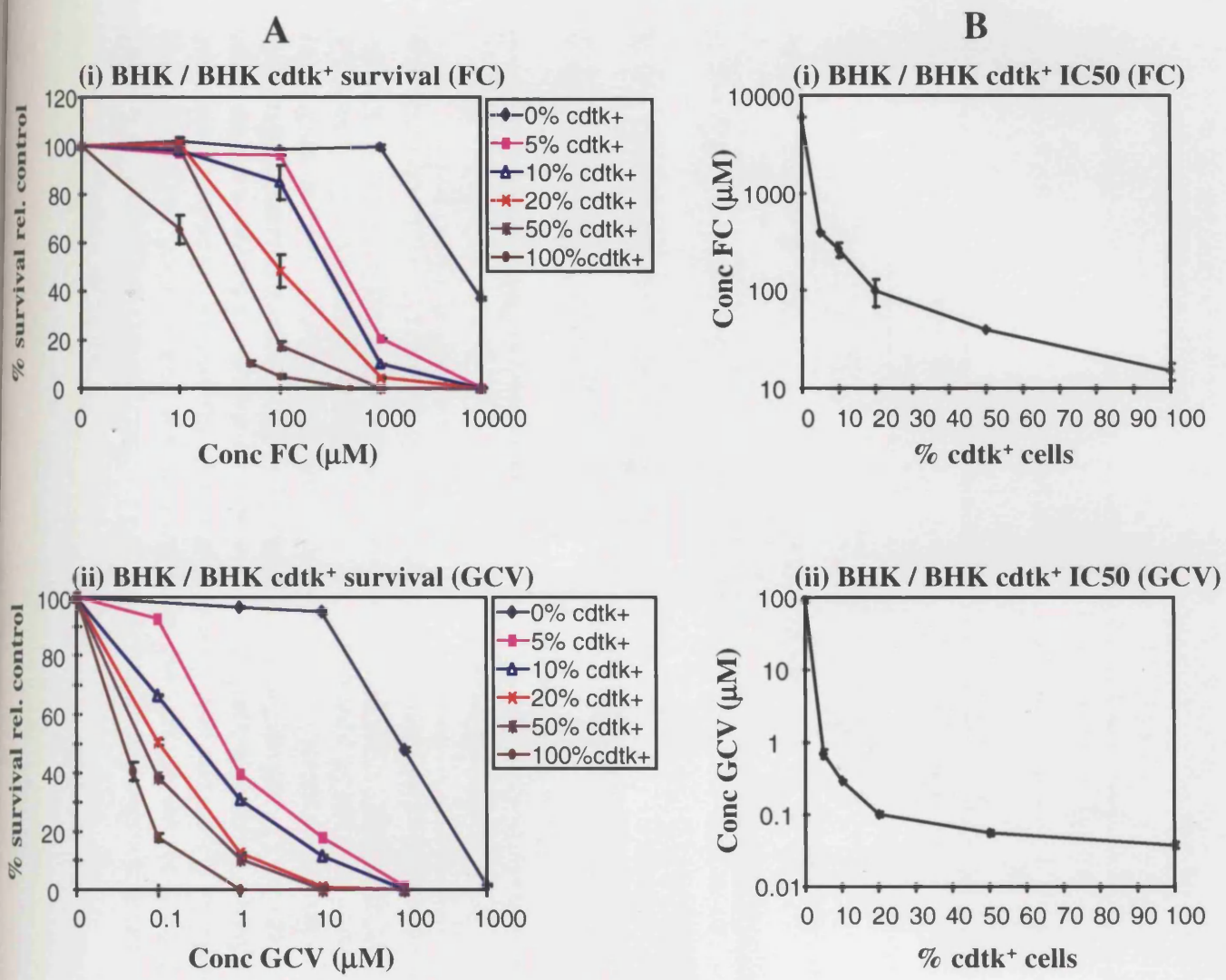


Figure 6.4. BHK cells transfected with a cdtk chimera are sensitive to FC and GCV and show bystander effects. **(A)** Varying mixtures of BHK cdtk+ and BHK cells were seeded (5 x 10^4 cells / ml) in 24 well tissue culture plates. 24 hr later cells were treated with FC **(Ai)** or GCV **(Aii)** for 2 days. After this time cells from each well were released with trypsin. Aliquots of the suspension were transferred to 96 well plates containing media and FC or GCV, as described in section 2.2.7. Survival was determined using the MTT assay. By calculation from each of the survival curves in **(A)**, the IC50, with respect to FC **(Bi)** or GCV **(Bii)**, was calculated. Each data point represents the mean +/_ SEM (bars) of two independent experiments, each performed in triplicate.

Table 6.1. Summary of characteristics of BHK cells transfected with a cdtk fusion construct and BHK cells transfected with the cd gene, then tk gene. Population doubling time was determined as described in section 2.2.5. Data represent mean ± SE from 2 experiments. GJIC has been determined by Lucifer Yellow dye injections as described in section 2.2.12. Data are from a minimum of 10 separate, 2 minute, injections and represent the mean ± SE. Specific enzyme activity has been determined by cd or tk specific assays (sections 2.4.3.; 2.4.4.). Activity represents pmoles ^{3}H cytosine converted / min / mg protein for cd and pmoles ^{125}I Iododeoxycytidine converted / hr / mg protein for tk. Data represent mean ± SE of the number of experiments shown in parentheses. Untransfected cells had undetectable levels of cd activity and tk activity of less than 0.2 pmoles ^{125}I Iododeoxycytidine converted / hr / mg protein. IC50s have been calculated from survival curves derived from treatment of mixed cultures of untransfected and transfected cells with FC or GCV. Bystander effect and therapeutic index have been determined from IC50s, as indicated in the table. Data represent mean ± SEM of two independent experiments each performed in triplicate.

Cell line	(A) BHK cdtk$^+$ fusion		(B) BHK cd$^+$-tk$^+$ double transfections	
Suicide gene system	**cd**	**tk**	**cd**	**tk**
Population doubling time (hr)	10.5 ± 0.5		11.5 ± 1	
GJIC	13.2 ± 2.5		12.0 ± 0.7	
Specific enzyme activity	1417 ± 95 (4)	14.4 ± 0.7 (4)	434 ± 31 (2)	10.4 ± 0.9 (4)
IC50 - 0% transfected cells	6050 ± 50	92.5 ± 7.5	6050 ± 50	92.5 ± 7.5
IC50 - 10% transfected cells	265 ± 45	0.3 ± 0.01	665 ± 125	0.85 ± 0.26
IC50 - 100% transfected cells	15 ± 3	0.038 ± 0.004	45 ± 2	0.05 ± 0.01
Bystander effect $^{(0\%\ IC50}/_{10\%\ IC50)}$	23.5 ± 3.8	314 ± 31	9.5 ± 1.9	118 ± 27
Therapeutic index $^{(0\%\ IC50}/_{100\%\ IC50)}$	421 ± 88	2441 ± 60	135 ± 5	1895 ± 228

Suicide gene	tk		tp		cd		cd-tk fusion			
							cd		tk	
Cell line	**Byst. Eff.**	**Ther. Ind.**	**Byst. Eff.**	**Ther. Ind.**	**Byst. Eff.**	**Ther. Ind.**	**Byst. Eff.**	**Ther. Ind.**	**Byst. Eff.**	**Ther. Ind.**
L929	1.04	3715	6.2	54	24.5	263	-	-	-	-
MCF-7	1.06	2242	-	-	-	-	-	-	-	-
CarB	351	1492	11.9	63	-	-	-	-	-	-
BHK	292	3530	12.1	116	6.3	109	23.5	421	314	2441
CarB-E	282	1134	-	-	-	-	-	-	-	-
B9	273	828	-	-	-	-	-	-	-	-

Table 6.2. Summary of bystander effects and therapeutic indic es in cell lines used to study the tk / GCV, tp / DFUR and cd / FC systems. Data have been collected from tables 3.1. (tk), 5.1. (tp), 5.2. cd and 6.1. cdtk. Bystander effects (Byst. Eff.) were calculated as $^{IC50\ 0\%\ gene+}$ / $_{IC50\ 10\%\ gene+}$ and therapeutic indic es (Ther. Ind.) as $^{IC50\ 0\%\ gene+}$ / $_{IC50\ 100\%\ gene+}$. All values represent the mean of two experiments each performed in triplicate.

picked and one of the clones that showed sensitivity to both FC and GCV was selected for further use.

GJIC and doubling times of this selected BHK cd^+-tk^+ clone were similar to the BHK $cdtk^+$ clone, but cd and tk activity were lower (compare Table 6.1.A. and B.). These differences in enzyme activities highlight the difficulty of obtaining clones with similar expression levels and such variations may be accounted for the number of integrated genes or whether the genes are inserted into silenced regions of the genome. The lower levels of expression generated weaker bystander effects and therapeutic indices in cultures containing BHK cd^+-tk^+ cells, in response to FC or GCV, compared to those with BHK $cdtk^+$ cells. However, comparison of the synergistic effects of BHK cd^+-tk^+ double transfectants with BHK $cdtk^+$ cells should not be affected by the proportional differences, since synergy should rely on increased concentrations of toxic metabolites produced by two, relative to one, enzyme prodrug systems. A lower expression of each enzyme will require higher concentrations of prodrug to generate a bystander effect, but combining the systems should still lead to an increased spread of toxicity.

To compare the above systems, survival of mixed cultures of 10% BHK $cdtk^+$ or 10% BHK cd^+-tk^+ cells and 90% BHK cells was measured after treatment with FC and GCV, as described previously (section 6.2.; section 2.2.7.). As before (section 6.2.), high or low concentrations of prodrug, separately or combined, did not result in significant differences in survival, but at intermediate concentrations of FC and GCV, combined treatment killed 15-20% more cells. This increase was similar in cultures with BHK cd^+-tk^+ or BHK $cdtk^+$ cells (figure 6.5.).

This demonstrates that a cdtk fusion protein can be used to combine these enzyme / prodrug systems and, in some cases, an enhanced bystander effect can be produced. The synergistic effect, seen in these experiments, occurred at intermediate prodrug concentrations

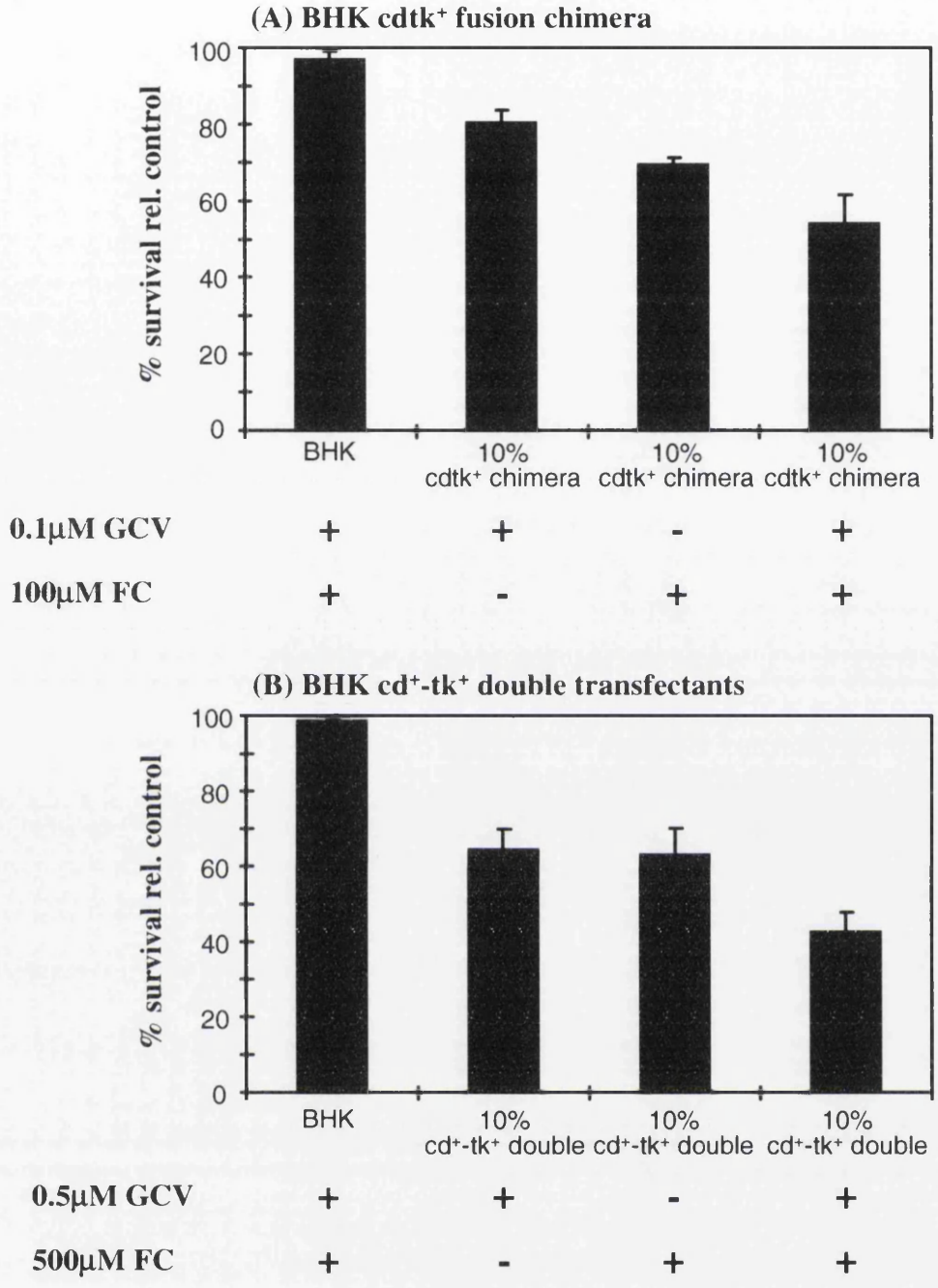


Figure 6.5. Combining the tk / GCV and cd / FC enzyme / prodrug systems results in an improved bystander effect at intermediate prodrug concentrations. 10% BHK cdtk$^+$-chimera **(A)** or 10% BHK cd$^+$-tk$^+$ double transfected **(B)** cells were mixed with 90% parental cells in 24 well plates (5 x 10^4 cells / ml). 24 hr later either GCV, FC or both were added to the media at the concentrations indicated in the graphs above. Survival was assayed using standard protocol (see section 2.2.7.) and expressed as percentage growth inhibition of untreated cells. All data represent mean +/- SEM (bars) of two independent experiments, each performed in triplicate.

and the level of synergy was similar when enzymes were expressed as a fusion protein or individually in the same cell.

6.4. Efficacy of the cdtk fusion protein *in vivo*.

An enhanced bystander effect was seen with combined FC and GCV treatment of cultures containing 10% BHK cdtk$^+$ cells and it is important to determine whether this improved killing leads to increased tumour regression *in vivo*.

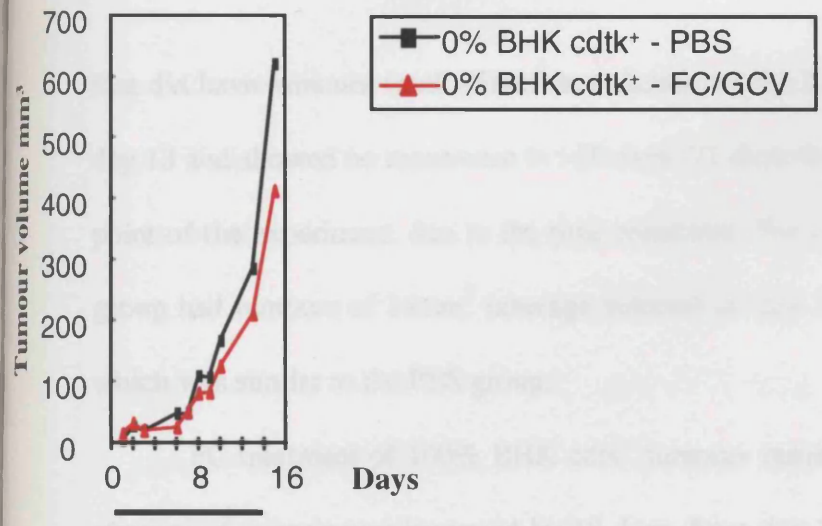
Tumours were generated in nude mice using BHK (0% BHK cdtk$^+$), BHK cdtk$^+$ (100% BHK cdtk$^+$) or mixtures of the two cell types (10% BHK cdtk$^+$) in the same way as described previously (section 4.2.; section 2.3.). There were 4 animals in each of the 10 treatment groups, which are described in Table 6.3. When tumours were approximately 4 mm in diameter, prodrug treatment was initiated. FC (250mg / kg) and / or GCV (45mg / kg) were administered i.p., daily, for 14 days. Survival was scored as tumour volume of <500mm^3 and animals exceeding this burden were terminated.

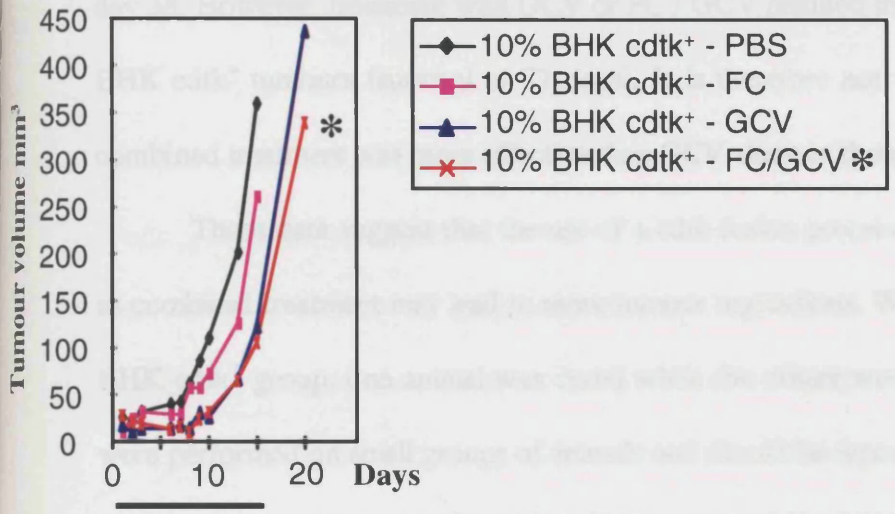
Animals with 0% BHK cdtk$^+$ tumours survived for a similar time with (FC / GCV) or without (PBS) treatment and this showed that combined prodrug had no effect on untransduced cells (figure 6.6.). Comparing the growth rates of BHK and BHK cdtk$^+$ cells showed that, although similar in culture, BHK cdtk$^+$ tumours grew more slowly *in vivo*. Survival of animals with 0% BHK cdtk$^+$ (PBS treated) tumours was only 15 days, but animals with 100% BHK cdtk$^+$ (PBS treated) tumours, survived for 20 days (see section 6.5. for further discussion).

With 10% BHK cdtk$^+$ tumours, survival was similar between PBS and FC treatment groups but was marginally extended in the GCV and in most of the FC / GCV treated animals (figure 6.6.). There was one exception, where an animal in the FC / GCV treatment group,

Group	Ratio BHK cdtk$^+$: BHK cells	Treatment
1	0% : 100%	PBS
2	0% : 100%	FC + GCV
3	10% : 90%	PBS
4	10% : 90%	FC
5	10% : 90%	GCV
6	10% : 90%	FC + GCV
7	100% : 0%	PBS
8	100% : 0%	FC
9	100% : 0%	GCV
10	100% : 0%	FC + GCV

Table 6.3. Determination of the relative efficacy of single or combined prodrug treatment on tumours containing different percentages of BHK cdtk$^+$ cells. Monolayers of BHK cdtk$^+$ or parental cells were trypsinised and viability assessed by trypan blue dye exclusion. Cell suspensions (10^6 / ml), at the ratios described above, were prepared. Nude mice (4 per group) were injected subcutaneously to each flank with 100µl of the suspension. Tumours were allowed to form for ~10 days before treating with FC (250mg / kg) and / or GCV (45mg / kg) intraperitoneally, once daily for 14 days, as described above. Results are shown in figure 6.6.





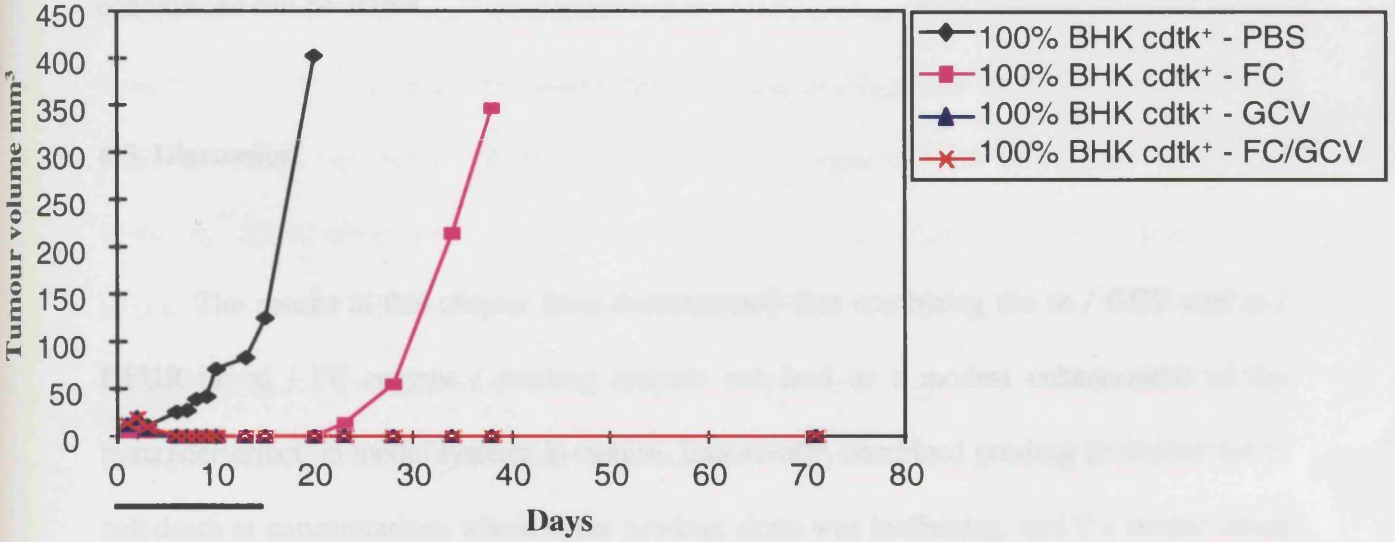


Figure 6.6. *In vivo* analysis of tumours containing different percentages of BHK cdtk+ cells. Nude mice were injected s.c. with BHK and BHK cdtk+ cell mixtures as described in figure 6.3. and section 6.4. The bar indicates the 14 day period of intraperitoneal injections of prodrug. Approximate tumour volumes were calculated from an average of two measurements ($^4/_3\pi r^3$). The different graphs are plotted against the same time scale. Animals were removed from the study if the total tumour burden exceeded 500mm^3. * In the 10% BHK cdtk+ group treated with FC and GCV, the tumours in one animal regressed and this animal was tumour free for >50 days (71 days total survival).

that did have tumours (each 35mm^3 in volume) on day 3, became completely tumour free by day 13 and showed no recurrence in >50 days (71 days total; figure 6.6.). Day 71 was the end point of the experiment, due to the time constraint. For comparison, the other animals in this group had tumours of 14mm^3 (average volume) on day 3, which grew to 80mm^3 by day 13, which was similar to the PBS group.

FC treatment of 100% BHK cdtk$^+$ tumours resulted in regression, but not cure, and survival of animals was increased by 18 days, from day 20, in controls treated with PBS, to day 38. However, treatment with GCV or FC / GCV resulted in cure of all animals with 100% BHK cdtk$^+$ tumours (survival of 71 days). It is therefore not possible to determine whether combined treatment was more effective than GCV alone in these 100% BHK cdtk$^+$ tumours.

These data suggest that the use of a cdtk fusion protein should be investigated further as combined treatment may lead to more tumour regressions. With the FC / GCV treated 10% BHK cdtk$^+$ group, one animal was cured while the others were not. However, these studies were performed on small groups of animals and should be repeated on larger groups. Since all animals survived in the GCV or FC / GCV treated 100% BHK cdtk$^+$ groups and only limited conclusions can be drawn.

6.5. Discussion

The results in this chapter have demonstrated that combining the tk / GCV and tp / DFUR or cd / FC enzyme / prodrug systems can lead to a modest enhancement of the bystander effect, in model systems in culture. Importantly, combined prodrug treatment led to cell death at concentrations where either prodrug alone was ineffective, and if a similar effect is seen in tumours, then cells will be in exposed to toxic levels of prodrug for longer periods of time.

Studies in nude mice have shown that a single daily intraperitoneal injection of 500mg / kg of FC produces a maximum plasma concentration of 4000μM and that the half life of FC is 40 minutes (Huber et al, 1995). Therefore the plasma concentration should be ≈100μM at 3 hr and undetectable at 6 hr. Culture studies in the previous sections have shown that in a population containing 10% BHK cdtk$^+$ cells, the IC50 for FC was 250μM. If this value applies to tumours in nude mice, then cells will have been exposed to prodrug at or above the IC50 dose for <3 hr each day. It is possible that this could explain the lack of tumour regression in the 10% BHK cdtk$^+$ group treated with FC.

Treatments with other prodrugs, such as GCV, are also likely to suffer from the problem of short term toxic levels (Markham and Faulds, 1994) and enhancing sensitivity to prodrug may be an important factor in achieving tumour cure. Furthermore, the prodrug concentrations that can be used in humans are often lower than those used in animal models and sensitivity to reduced levels of prodrug may be all the more important.

The tk / GCV system has also been combined with the nitroreductase (ntr) / 5-arizidin-1-yl-2,4 dinitrobenzamide (CB1954) system (Bridgewater et al, 1995). CB1954 is converted, by ntr, to a toxic compound (5-arizidin-1-yl-4-hydroxyamino-2-nitrobenzamide) that cross-links DNA and toxicity is not dependent on cell cycling (Bridgewater et al, 1995). In NIH3T3 cells, combined expression of the tk and ntr genes enhanced the therapeutic index and bystander effect when prodrug treatment was given in combination. Enhancement of cell killing was optimal when cells were exposed to GCV for 72 hr and to CB1954 for 24 hr. In the studies here, the combined effect of the tk / GCV and tp / DFUR systems was most effective when prodrugs were added simultaneously. For treatment of tumours with different combined enzyme / prodrug strategies, the optimal timing and application of prodrug will need to be established.

The results here show that the arrangement of enzymes within a fusion protein is important but the consequences cannot be predicted. For example, colonies that were sensitive to FC and GCV could be selected from transfections with the cdtk construct, but not from those with the tkcd construct. The lack of prodrug sensitivity in tkcd transfectants was not expected, on the basis of earlier work with fusion proteins containing tk or cd. In tk-neo (Veelken et al, 1996) or CD44-cd (Asman et al, 1995) fusion chimeras, the suicide enzymes were functional even though folding of the C-terminus of tk or the N-terminus of cd was restricted by the presence of another peptide domain. In the tkcd fusion protein described here, the folding of the same termini were restricted, but there was no detectable sensitivity, of G418 selected colonies, to GCV or FC.

Most BHK cdtk$^+$ clones isolated were sensitive to GCV but only 20% were sensitive to FC and for a more detailed study, the clone selected had a high level of cd activity. If, however, the clones had been pooled, cd activity would have been reduced since a large percentage were not sensitive to FC. Under these conditions, the results appear consistent with another report that was published during the course of this work. Rogulski et al (1997) constructed a cdtk fusion protein where the two enzymes were separated by 11 residues (10 glycines and one phenylalanine), which to distinguish it, will be termed cd-gly-tk. Retroviral transduction with the cd-gly-tk fusion construct (and neo) of 9L cells resulted in pooled clones having 40% cd- and 200%-tk activity compared to transduction of the same cells, with genes in separate vectors. The predicted and actual lower activity of cd in the cdtk and cd-gly-tk chimeric proteins, respectively, may be accounted for by incorrect folding of cd, which could be disturbed if the N-terminus of tk forms a more stable structure, or by stearic hindrance, which could arise as a result of the position of the tk domain in relation to the active site of cd. Both these suggestions could be correct, but until the positions of the active sites are determined, this can only be speculation.

Although the study by Rogulski did not analyse the bystander effect, it demonstrated that pooled 9L cd-gly-tk clones were more sensitive to GCV than FC, and combining intermediate concentrations of each prodrug enhanced cell killing, in culture. These observations support the data in this thesis.

Treatment of animals with 100% BHK cdtk$^+$ tumours with GCV for 14 days led to tumour cure in all mice (this chapter), whereas the same regime, for only 7 days, did not cure animals with 100% BHK tk$^+$ tumours (chapter 4). It is possible that increasing the length of prodrug treatment led to the increased rate of tumour cure and such a suggestion is supported by the observations of others (Osaki et al, 1994). There may, however, be alternative explanations for the cures seen, for example, the slower *in vivo* growth rates of BHK cdtk$^+$ cells compared to BHK tk$^+$ cells, or the presence of the cdtk fusion protein.

Slower growth of cells *in vivo* may mean that at the start of prodrug treatment the volume of 100% BHK cdtk$^+$ tumours was less than that of the 100% BHK tk$^+$ tumours, which could account for the cures seen in the former group. However, the volumes of tumours within these different groups were similar at the time of treatment, and a slower growth rate, if anything, would be expected to reduce animal survival since GCV mediated killing requires cells to be cycling. Support for this suggestion comes from a study where groups of animals were inoculated with 9L tk$^+$ cells, with high or low rates of proliferation *in vivo*, then treated with GCV (Iwadate et al, 1996). Survival was inversely proportional to tumour growth rate, i.e. the animals with the fastest growing tumours survived the longest.

Enhanced eradication of tumours may have been due to the presence of the cdtk fusion protein, rather than tk alone. This possibility cannot be excluded since the fusion protein may lead to greater activation of, for example, macrophages or granulocytes (T-cells are absent from nude mice). Previously it has been suggested that the antigenicity of the cd suicide

protein may be an important factor in the clearance of tumours in syngeneic mice (Mullen et al, 1996).

The cure of one 10% BHK cdtk$^+$ tumour bearing animal, but not the other three, suggests that this fusion chimera should be investigated further to establish whether combining the cd / FC and tk / GCV enzyme / prodrug systems could be used as a potential treatment in cancer therapy. Combining other systems may be warranted and possible candidate genes are discussed in the next chapter.

Chapter 7. Discussion and future directions.

Enzyme / prodrug systems represent a novel approach for the treatment of cancer. The use of the tk / GCV strategy has led to decreases in tumour size in some patients with glioblastoma multiforme, but although this is encouraging, treatment had no effect in the majority of patients (Culver et al, 1996; Izquierdo et al, 1996). The lack of therapeutic effect was most likely due to the low level of transduction (<0.2%) and the limited number of cells in S-phase (Ram et al, 1995). Clearly, if suicide gene therapy is to be considered for use in cancer treatment, cell killing needs to be improved. The primary aim for improvement must be the enhancement of gene transfer, since gene expression in the target cells is a fundamental requirement for enzyme / prodrug systems. Enhancing gene delivery is an area that is currently being addressed (e.g. Kruse et al, 1997). However, a better understanding of the mechanism of bystander effects of enzyme / prodrug systems may also lead to improved treatment by facilitating the choice of the most appropriate regime for each cancer.

In this thesis the mechanisms of the bystander effects in the tk / GCV, tp / DFUR and cd / FC strategies have been investigated. Results from analysis of the tk / GCV system have shown that gap junctions are required for the spread of toxicity from tk^+ to tk^- cells, and when coupled, even at a low level, high levels of tk activity result in extensive bystander killing. However, if the level of tk activity, or the level of communication is sufficiently low, then increasing coupling, by introduction of genes involved in gap junction formation, may enhance spread of toxicity.

There are now several reports that document an increase in tk / GCV bystander effect, in culture, when communication of poorly coupled cells is increased by transfection of connexin genes (Mesnil et al, 1996; Elshami et al, 1996; Mesnil et al, 1997; Dilber et al, 1997; Shinora et al, 1996; Vrionis et al, 1997). These reports did not examine the levels of tk

activity, and it is possible that tk expression was low. This would contrast to the high levels of tk activity in the cell lines described in this thesis, which may account for the failure to enhance the bystander effect in poorly coupled CarB cells, by increasing the level of coupling by transfection with E-cadherin (Chapter 3; Holden et al, 1997)

Experiments *in vivo* have demonstrated that tumours formed from tk^+ and tk^- C6 glioma cells are cured more often when Cx43 expression had been increased by transfection (Dilber et al, 1997). These observations suggest that introduction of genes involved in gap junction formation may enhance the tk / GCV bystander effect in some human tumours. To employ this approach, tumours will have to be characterised, and those with low levels of coupling and connexin expression, may be suitable for treatment.

On the basis of the data obtained in this thesis, another way to enhance the tk / GCV bystander effect may be to increase levels of tk expression. Results in chapter 3 demonstrated a correlation between bystander killing and level of tk activity, but it seems likely that the use of tissue specific promoters, to prevent ubiquitous expression of the therapeutic gene, will reduce the level of enzyme activity (Kanai et al, 1997). Methods of increasing tk activity, not involving promoters, are likely to be required.

One possibility may be to modify the structure of the tk protein so that activity is enhanced. Such an approach, based on analysis and subsequent modification of the 3 dimensional structure, has proved successful in enhancing the activity of glucoamylase, from *Aspergillus awamori* (Fierobe et al, 1996). However, nuclear magnetic resonance or x-ray crystallograpy data are not yet available for the tk enzyme, and the 3 dimensional structure of tk is not known. Furthermore, computer modelling predictions of the residues involved in substrate binding have not been consistent with mutational analyses (Folkers et al, 1991; Michael et al, 1994).

An approach that has had some success is the generation of mutant forms of the tk enzyme. Munir et al (1994) produced 190 tk mutants, by modifying a portion of the nucleotide sequence that encodes a putative thymidine binding site, and showed that one mutant had a five fold higher activity towards phosphorylation of thymidine. Although this study did not investigate phosphorylation of GCV, it shows that this is a potentially feasible, even if labour intensive, way to enhance activity. However, activity may not be the only factor that should be taken into consideration. If mutants with increased activity are used, the strength of substrate binding ($^{1}/_{Km}$) versus the rate of the reaction (K_{cat}) should be considered. It has been suggested that enzymes with the lowest K_m (strongest binding) and the highest K_{cat} (highest rate of activity) may not be ideal choices, since cells closest to the blood vessels would convert all prodrug to drug, without allowing prodrug to penetrate to cells positioned deeper within the tumour (Connors and Knox, 1995). Penetration of the prodrug to the tumour is likely to be a requirement in the therapies that are discussed later.

Enzyme / prodrug systems that involve tk, tp or cd suicide genes are most effective at killing when cells are in S-phase of the cell cycle, which means that their use may be limited to fast growing tumours, such as some pituitary tumours (Anniko et al, 1983). A large percentage of the cells of many tumours, for example, malignant glioma (Nishiyama et al, 1985), are cycling slowly, or out or cycle, and treatment is likely to be more effective if the therapy does not require cells to be in cycle.

The ntr / CB1954 system converts the prodrug to a bifunctional alkylating agent that kills cycling and non-cycling cells by introducing DNA interstrand cross links (Sunters et al, 1991). A potential advantage of this system is that ntr is the only enzyme required for the formation of the toxic metabolite and further reactions catalysed by cellular enzymes, which could be rate limiting, are not involved. This contrasts to the systems described above, where a

minimum of two other reactions are required for toxicity, and in some instances, these may be rate limiting (see discussion of Chapter 3). The ntr / CB1954 system appears promising from culture studies, where a therapeutic index of nearly 1000 has been demonstrated (Bailey et al, 1996). However, experiments in transgenic mice, where expression of ntr is restricted to T-cells (Drabek et al, 1997) or to the luminal cells of the mammary gland (Clark et al, 1997), suggest that the difference in sensitivity between toxic doses of prodrug administered systemically and doses required to kill the ntr^+ cells may be less than 5-fold. Another possible draw-back is the apparent lack of bystander effect in some (Clark et al, 1997), but not other (Bridgewater et al, 1997) experimental models. It is possible that a more effective prodrug will be synthesised from on-going studies (Anlezark et al, 1995).

An alternative to the ntr / CB1954 system is the use of the E.coli DeoD gene, which codes for purine nucleoside phosphorylase (PNP). This enzyme converts the prodrug 6 methylpurine-2'-deoxyribonucleoside (MP-d) to the toxic adenine analogue, 6 methylpurine (MP) which inhibits protein and RNA synthesis (Sorscher et al, 1994). Killing is not dependent on the cells being in cycle, and the bystander effect seen is transferable via the medium (Hughes et al, 1995). This PNP / MP-d system appears promising and inclusion in a proposed treatment regime is discussed later.

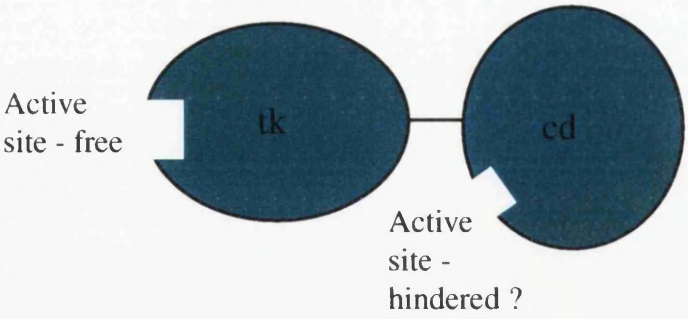
The results in chapter 6 demonstrated that combining cd and tk systems can lead to an enhanced bystander effect and the improvement can be achieved with a fusion protein. However, Rogulski et al (1997) showed that cd activity of 9L cells expressing a cd-gly-tk fusion chimera was reduced when compared to 9L cells that expressed cd alone and this appears to be consistent with the data in chapter 6 (see section 6.5.). If cd activity is reduced due to incorrect folding of cd in the chimeric protein, then it is possible that a linker region that is longer, or comprised of different amino acids, may increase cd activity. However, with the exception of the cdtk and cd-gly-tk fusion proteins described here and by Rogulski et al

(1997), there are no available reports that document the fusion of a peptide domain to the C-terminus of cd, therefore modification of the linker region cannot be based on previous experience using fusion chimeras involving this enzyme.

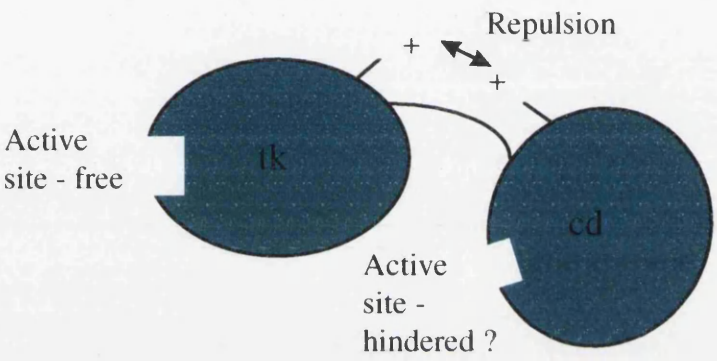
If cd activity is reduced by stearic hindrance of the active site by the tk domain, again, modifying the linker region may be an advantage (figure 7.1.). In the fusion protein described in this thesis, the cd and tk enzymes were separated by two amino acids and the close proximity of the enzymes may lead to direct hindrance (figure 7.1.A.). In the report by Rogulski, separation was achieved with 11 residues, most of which were glycine so the linker region is likely to be flexible. If amino acids on the surface of each enzyme cause repulsion (or attraction), this may lead to stearic hindrance (figure 7.2.B.) which might be increased with a flexible linker region. If these hypotheses are correct, then introducing a structured linker region, such as an α-helix, may be of benefit (figure 7.1.C.). Whether such a structure would be stable and lead to enhanced enzyme activities, would need to be tested. If changes in the linker region were successful in raising the level of activity, similar techniques could be applied to the fusion proteins discussed later.

Combining treatments can, under the correct conditions, lead to an enhanced therapeutic effect. For example, 9L cells expressing a cd-gly-tk fusion are more effectively killed when FC, GCV and γ-irradiation (^{137}Cs source) treatments are given together, compared to any other separate or combined (dual) treatment using these components (Rogulski et al, 1997). However, careful consideration is required when different treatments are combined, since killing could be reduced if antagonistic therapies are used. For example, tk expression from an AFP-tk construct was increased in the presence of dexamethasome, leading to enhanced cell killing (Ido et al, 1995), but *in vivo*, this steroid suppresses the immune response (Barba et al, 1994; Ram et al, 1994). Dexamethasome might be expected to reduce an immune mediated bystander effect but, never the less, has been used to reverse GCV induced

7.1.A. - short linker region



7.1.B. - flexible linker region



7.1.C. - structured linker region

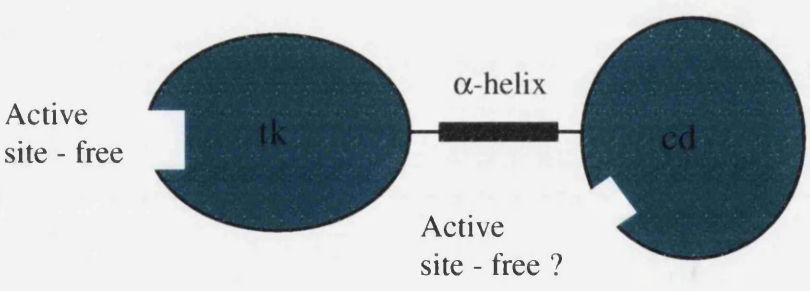


Figure 7.1. Hypothetical role of the linker region between fusion proteins.

neutropenia in clinical trials using the tk / GCV system (Culver, 1996). This observation suggests that alternative therapies such as systemic treatment with the cytokine, granulocyte colony stimulating factor (GCSF), might be used to alleviate symptoms (Markham and Faulds, 1994).

In a different example, combined use of FU and verapamil together can be beneficial (Shchepotin et al, 1994) but verapamil inhibits the tk / GCV bystander effect (Marini et al, 1996). The reduction in bystander killing may occur through reduced levels of GJIC. Verapamil inhibits calcium efflux from the cell (Taylor and Simpson, 1992) which could result in increased concentrations of intracellular calcium ions, a factor that may be associated with a reduction in junctional communication (review, Loewenstein, 1979). Combining verapamil with FC and GCV treatment in cdtk transduced cells would, therefore, not be desirable.

Most tumours consist of dividing and non dividing cells. Therefore, combining suicide gene systems that have proved to be effective at killing cells that are in S-phase (e.g. the tk / GCV and cd / FC systems) with those that provide the opportunity to also kill non dividing cells (e.g. the PNP / MP-d and ntr / CB1954 systems), may be beneficial. However, due to the importance of the immune system, any therapy that does not enhance immune infiltration is unlikely to succeed on a regular basis. There are now several reports that suggest combining suicide gene systems and cytokine gene delivery can lead to an increase in tumour cures in experimental animals (Chen et al, 1995b; Chen et al, 1996b; Mullen et al, 1996; Santodonato et al, 1996). It may therefore be advantageous to combine the introduction of several suicide and cytokine genes. The final pages of this thesis are included as a suggestion for the development of a theoretical strategy to try to accomplish such a combination. A summary is provided in figure 7.2. and the logic behind the suggested strategy is provided below.

Cytokines are secreted into the extracellular space, where they bind to and activate the proliferation of particular cell lineages of the immune system. For example, IL-2 is required for

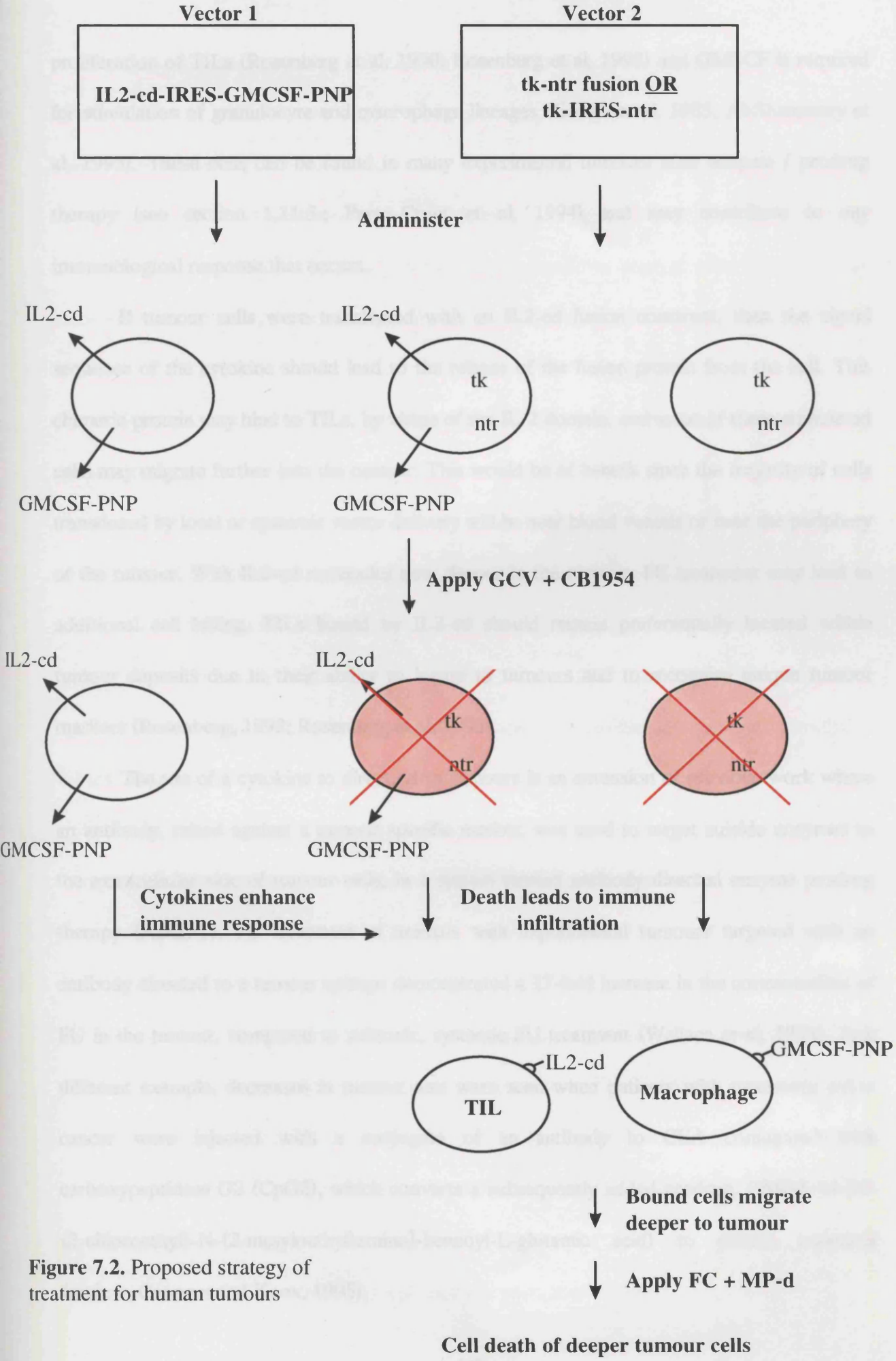


Figure 7.2. Proposed strategy of treatment for human tumours

proliferation of TILs (Rosenberg et al, 1990; Rosenberg et al, 1993) and GMSCF is required for stimulation of granulocyte and macrophage lineages (Gough et al, 1985; Al-Shammary et al, 1995). These cells can be found in many experimental tumours after enzyme / prodrug therapy (see section 1.11.5.; Perez-Cruet et al, 1994) and may contribute to any immunological response that occurs.

If tumour cells were transfected with an IL2-cd fusion construct, then the signal sequence of the cytokine should lead to the release of the fusion protein from the cell. This chimeric protein may bind to TILs, by virtue of the IL-2 domain, and some of these stimulated cells may migrate further into the tumour. This would be of benefit since the majority of cells transduced by local or systemic vector delivery will be near blood vessels or near the periphery of the tumour. With IL2-cd molecules now deeper in the tumour, FC treatment may lead to additional cell killing. TILs bound by IL2-cd should remain preferentially located within tumour deposits due to their ability to locate to tumours and to recognise unique tumour markers (Rosenberg, 1992; Rosenberg et al, 1993).

The use of a cytokine to direct cd to tumours is an extension of previous work where an antibody, raised against a tumour specific marker, was used to target suicide enzymes to the extracellular side of tumour cells, in a system termed antibody directed enzyme prodrug therapy (ADEPT). FC treatment of animals with experimental tumours targeted with an antibody directed to a tumour epitope demonstrated a 17-fold increase in the concentration of FU in the tumour, compared to subtoxic, systemic FU treatment (Wallace et al, 1994). In a different example, decreases in tumour size were seen when patients with metastatic colon cancer were injected with a conjugate of an antibody to CEA conjugated with carboxypeptidase G2 (CpG2), which converts a subsequently added prodrug, CMDA (4-[N-(2-chloroethyl)-N-(2-mesyloethyl)amino]-benzoyl-L-glutamic acid) to phenol mustards (review, Connors and Knox, 1995).

The disadvantage of the ADEPT approach is that active conjugate remains in the blood, which can lead to conversion of prodrug to drug and hence general toxicity. Adverse effects can be reduced, but not prevented, by introducing a clearing antibody which binds to the active site of the enzyme and decreases the level of active circulating conjugate (review, Connors and Knox, 1995). Targeting the cDNA for CpG2 to tumour cells, for use as an intracellular enzyme / prodrug system, is now being investigated in culture (Marais et al, 1996).

The use of extracellular targeting will place limitations on the enzyme / prodrug systems that can be used, because strategies that require, for example, ATP or a reducing environment for prodrug activation, will not be effective. The toxic metabolites produced must also be able to cross the membrane to enter cells. Therefore, fusions between a cytokine and tk (requires ATP to produce GCV-MP) or ntr (requires NAD[P]H as a cofactor) cannot be used. It is possible that the characteristics of the cd / FC and PNP / MP-d systems, such as a bystander effect mediated by membrane permeable toxic metabolites, may make them suitable for this strategy.

Based on previous results (Chapter 6), it is likely that the proposed system would be enhanced if two cytokine-enzyme fusions were introduced to the tumour, and suitable choices may be IL2-cd and GMCSF-PNP (figure 7.2.). However, there will be a limit to the number of viral particles that can be introduced to patients before toxicity arises and fusion constructs should, preferably, be contained within the same vector but separated by an IRES site to avoid problems of promoter interference (Chapter 6; Emmerman and Temin, 1984). Previous studies on translation of peptides from bi- and tri-cistronic vectors, containing single or fused constructs, has shown this approach to be successful, in culture (Tahara et al, 1995; Okada et al, 1996; Veelken et al, 1996; Lotze et al, 1997; Rogulski et al, 1997), which suggests that expression of two fusion proteins, by a similar approach, may be feasible (figure 7.2.).

The level of immune activity in spontaneously arising tumours is low (Sacco et al, 1996) and transfer of fusion constructs by TILs or granulocytes into deeper regions of the cancer is unlikely to occur. In addition, the few cells of the immune system that are present may be destroyed by prodrug treatment, which could suppress the immune mediated bystander effect. Immune infiltration may be enhanced if a second vector, carrying only suicide genes, was introduced (figure 7.2.).

Previous reports have demonstrated that the tk / GCV system can lead to activation of an immune response in tumours (Perez-Cruet et al, 1994). Again, including two suicide genes may produce a greater effect and these could be expressed as two peptides from a bicistronic message or as a single fusion protein. The enzymes used in this second vector should activate different prodrugs to the cytokine-enzyme fusion proteins, so that immune stimulation and the effect of further prodrug treatment are not attenuated (figure 7.2.). Potential choices may be the tk and ntr genes.

To apply this strategy, the timing of administration of vectors 1 and 2 (figure 7.2.) would need to be optimised, as would that of each of the prodrugs. Simultaneous vector delivery may lead to transduction of the same or separate tumour cells, and it might be anticipated that transduction of the same cells would reduce the therapeutic effect (see above). If intratumoural or local vector delivery is used, such as implantation of retroviral vector producer cells (Oldfield, 1993), then different sites of implantation may reduce transduction of the same cells by vectors 1 and 2. Following tumour transduction, it may be possible to apply a protocol, along the lines of that outlined below.

Administration of GCV or CB1954 should kill some of the tk or ntr expressing cells and this should lead to immune infiltration, which may be enhanced by the presence of cytokine activity in IL2-cd and GMCSF-PNP fusion proteins (figure 7.2.). Some of the immune cells bound by the fusion chimeras may migrate deeper into the tumour and, it is

hoped that prodrug treatment, using FC and MP-d, would lead to additional killing of cycling and non cycling tumour cells (figure 7.2.).

The timing of FC and MP-d treatment will have to provide a compromise between the level of production of the fusion protein versus inactivation of the fusion chimera by host antibodies. It may be of benefit to combine treatment further with radio- or chemo-therapy, but due to the potential antogonistic effects of combinations, the choice will have to be considered carefully.

To employ a strategy based on these ideas and suggestions, the feasibility of several aspects will need to be evaluated. Many current efforts are aimed at increasing the titre of viral vectors to increase the transduction efficiency (Kaptein et al, 1997; Kruse et al, 1997), but introduction of larger amounts of foreign genetic sequence may reduce viral titres (review, Chong and Vile, 1996) and this would be expected to reduce the level of transduction. The strategy proposed here would require introduction of up to 4kb of gene sequence to vectors, not including 5'- or 3'- untranslated regions (such as promoter sequences).

There are no available reports that have documented the use of the PNP / MP-d strategy in an ADEPT approach and the extracellular activation of this prodrug would need to be established.

Previously in this thesis it was pointed out that the folding of fusion proteins, containing cd and tk suicide enzymes, could not be predicted, so it is not known whether the cytokine-suicide enzyme fusions suggested here would be functional. Furthermore, it may be difficult to determine how these fusion proteins will behave in the tumour environment, where pH and ion concentrations may differ to more normal physiological conditions (Denny, 1996). Another consideration is whether the immune cells will carry the cytokine-enzyme fusion chimeras deeper into the tumour bed, and even if this does occur, whether prodrug

concentrations, in these regions, will be sufficient to kill cells. It is possible that only a detailed investigation will resolve these issues.

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