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Gene-directed enzyme prodrug therapy (GDEPT): choice of prodrugs

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Abstract

Gene-directed enzyme prodrug therapy (GDEPT) is a two step gene therapy approach where the gene for a non-endogenous enzyme is directed to target tissues. The enzyme is expressed intracellularly where it is able to activate a subsequently administered prodrug. It is a promising new treatment for cancer chemotherapy. The design and synthesis of prodrugs able to undergo intracellular enzymic activation by foreign genes in such systems is an essential component. This review will focus on the requirements to be fulfilled by the prodrugs used in this system. A special emphasis is placed on the description of the prodrugs (antimetabolites and alkylating agents) used in GDEPT protocols. The bystander effect and its implications for the design of improved prodrugs for GDEPT is also discussed.

Keywords: GDEPT; Gene therapy; Prodrug; VDEPT

Contents

1. Introduction

Gene therapy may be broadly defined as a genetic technology aimed at modifying cells for therapeutic gain. In cancer gene therapy, both malignant and non-malignant cells may be targeted for a therapeutic benefit (See Scheme 1).

There are many proposals to modify genetically (or to suppress the expression of) tumour cells (reviewed in [1]). An early suggestion was the antisense oligonucleotide strategy which targets specific mRNA sequences (reviewed in [1]). Another approach is the 'antigene oligonucleotide' approach

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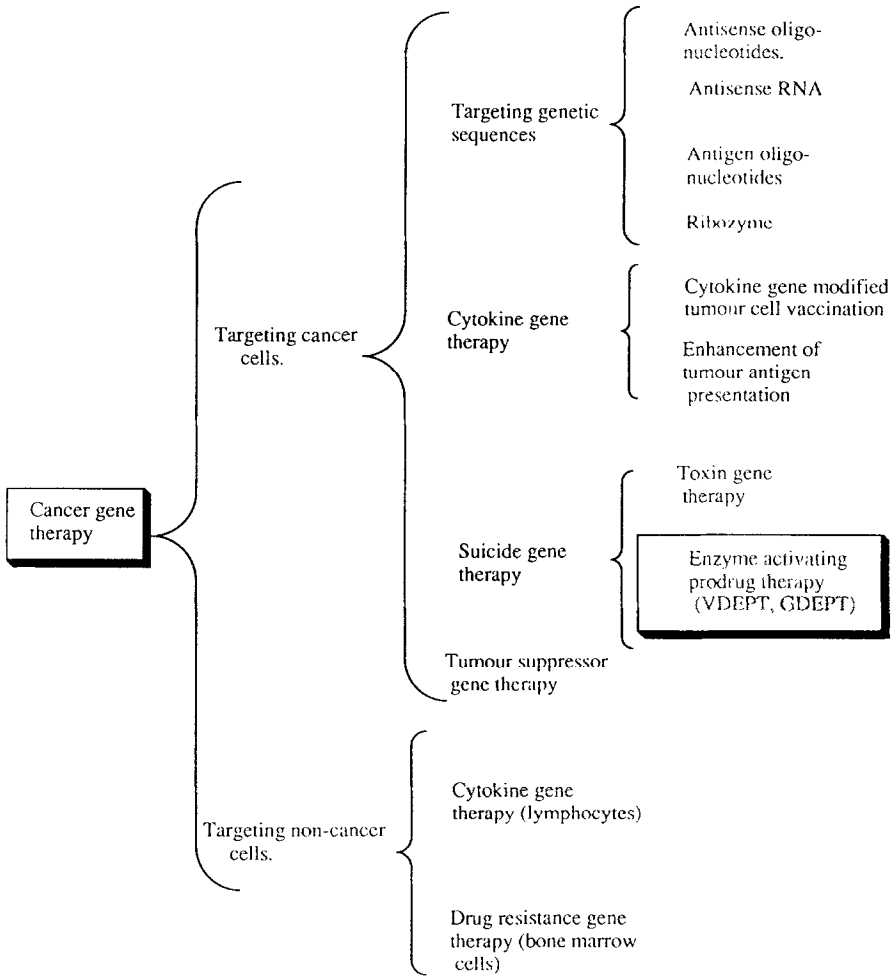


Scheme 1.

which aims to target DNA sequences (reviewed in [1]). Other strategies involve antisense RNA (which interferes with transcription and translation, blocks RNA splicing or induces double stranded DNA cleavage) and antioncogene ribozymes (reviewed in [1]). Also cytokine gene therapy has been proposed in an attempt to elicit an immune response generating antigenic tumour cells. In addition, introduction of tumour suppressor genes is also under consideration (reviewed in [1]). Finally, the possibilities to render cancer cells more sensitive to chemotherapy or toxins, either by suppressing the expression of resistances gene (e.g. MDR-gene) or by introducing 'suicide genes' are being considered (reviewed in [1]). The latter includes two approaches: toxin gene therapy whereby transfected genes are able to generate toxins and enzyme-activating prodrug therapy whereby transfected genes express foreign enzymes which can activate prodrugs inside the cancer cells. This latter approach is termed GDEPT (gene-directed enzyme prodrug therapy) or VDEPT (virally-directed enzyme prodrug therapy).

The purpose of this chapter is to review the progress made in the design and syntheses of the prodrugs involved in GDEPT and to discuss some of the theoretical and practical issues.

GDEPT is a two phase therapy. It requires:

1. a gene producing a non-endogenous enzyme (or an enzyme that is only expressed at a low level) which is able to activate a prodrug;
2. injection of the prodrug.

In order to achieve success in GDEPT a number of factors must be considered. Firstly, the gene should be expressed only in the tumour cells. Secondly expression in the cancer cells should be as high as possible. Unfortunately, when injected systemically this is unlikely to be greater than 10–20% of tumour cells in vivo. Therefore, a bystander effect is required whereby the prodrug is cleaved to an active drug which kills not only cells expressing the foreign enzyme but also cells that are not expressing enzyme. This means that less than 100% infection of cells can still give total cell kill. It has been demonstrated, in vivo, that when as few as 2% of the tumour cells are infected with foreign enzyme, then subsequently treated with suitable prodrug, long-term survivors have been obtained [2]. Therefore, an expression efficiency of 10–20% should be enough to achieve 100% cell kill in tumours and efficiencies of 1–5% are considered sufficient for a therapeutic response [2]. It was claimed that, even with lower transfer efficiencies, results can be obtained if the transduced cell population is allowed to expand.

2. Prodrugs used in GDEPT

An essential component is the prodrug/drug system. It must meet a number of requirements: (1) the prodrug must be able to cross the tumour cell membrane; (2) the cytotoxicity differential between the prodrug and its corresponding drug should be as high as possible; (3) the prodrug should be a good substrate for the expressed enzyme; (4) the active drug should be highly diffusible or be actively taken up by cells to achieve the bystander effect; (5) the active drug should be as cytotoxic as possible.

To date the literature contains five classes of prodrugs which have been described for GDEPT, namely:

1. 6-methoxy purine arabinonucleoside which is activated by the viral thymidine kinase (TK) [3];
2. purine nucleosides: acyclovir, ganciclovir and 1(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracyl which are activated by viral TK [4–14];
3. 5-fluorocytosine activated by bacterial cytosine deaminase (CD) [2,15–19];
4. cyclophosphamide and isophosphamide activated by rat liver cythochrome P-450 isoenzyme (CYP) [20];
5. CB 1954 activated by bacterial nitroreductase (NR) [21].

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

2.1. 6-Methoxy purine arabinonucleoside (ara-M)

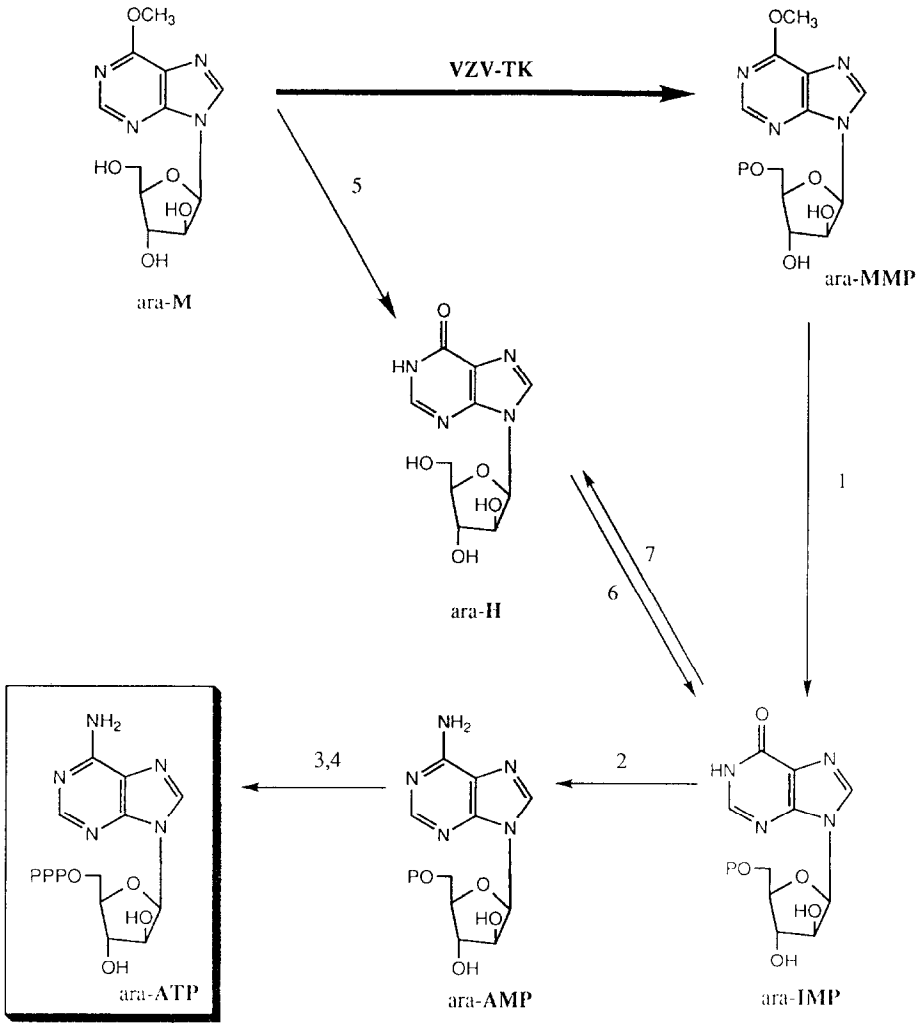
The basis for this prodrug choice was that ara-M is a good substrate for varicella zoster virus (VZV)-TK but a poor substrate for the three major mammalian nucleotide kinases [3]. The prodrug is activated by the viral enzyme to ara-M-monophosphate (ara-MMP) which is then further converted by the mammalian AMP-deaminase to ara-IMP. This metabolite is further catalysed by the mammalian adenylosuccinate synthetase lyase into ara-AMP which is then phosphorylated by the AMP-kinase and subsequently by the cellular nucleoside diphosphate kinases to the anabolite ara-ATP (see Scheme 2). Ara-ATP exerts its cytotoxic effects on dividing cells, by inhibiting DNA synthesis.

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

This approach has provided a therapeutic model for the treatment of hepatocellular carcinoma (HCC) [3]. This model takes advantage of the transcriptional differences between normal and malignant cells by using a replication defective retrovirus since normal hepatocytes are essentially non-dividing.

A chimeric gene was constructed which contained the protein coding domain of varicella zoster virus thymidine kinase (VZV-TK) under the control of the tissue specific regulatory sequence, hepatoma-associated α-fetoprotein (AFP) tissue regulatory sequence (TRS) or liver associated albumin (ALB) TRS [22]. If the TRS is from a tumour associated gene, than the chimeric gene will produce tumour specific expression of VZV-TK gene. These chimeric tumour and liver specific genes were placed in a replicative defective retroviral shuttle CR74 and CR78 viruses, respectively.

Human hepatoma Hep G2 cells (expressing high



Scheme 2. Metabolism of ara-M [3]. 1, AMP-deaminase; 2, adenylosuccinate synthetase lyase; 3, AMP-kinase; 4, nucleoside diphosphate kinase; 5, adenosine deaminase; 6, cellular kinases; 7, cellular phosphatases.

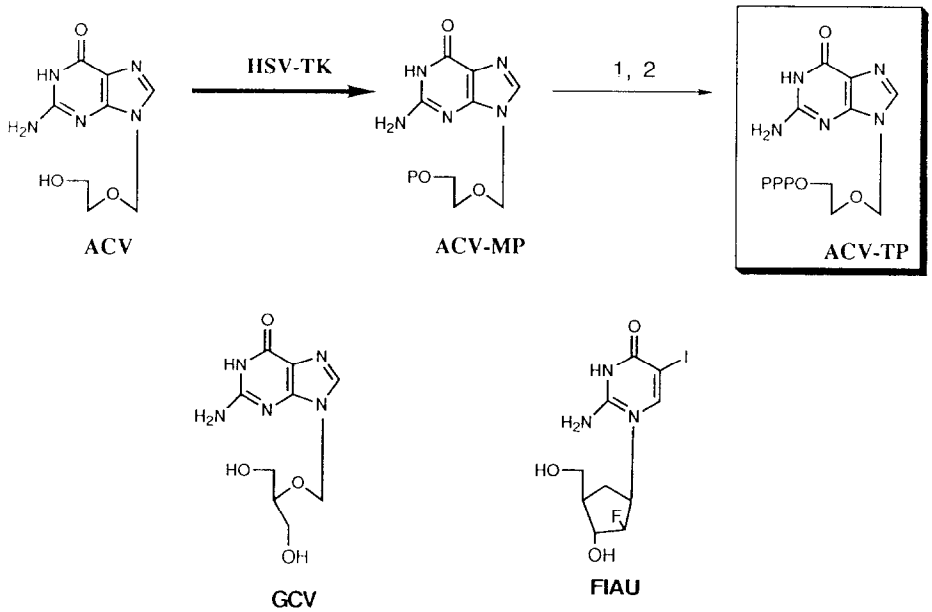
levels of both ALB and AFP) and HuH7 non-hepatoma cells (expressing high levels of AFP and moderate levels of ALB) were infected with both the CR74 and CR78 virus. In the control Hep G2 parental cells, the IC_{50} ratio of prodrug: drug was greater than 2000 (ara-M, $IC_{50} > 2000$ μg; araATP < 1 μg). After infection, the IC_{50} of ara-M was greatly decreased in Hep G2 cells ($IC_{50} = 6.5$ μg in Hep G2-CR74 cells and $IC_{50} = 3.1$ μg in Hep G2-CR78 cells) as well as in HuH7 cells ($IC_{50} = 11$ μg in Hep G2-CR74 cells and $IC_{50} = 36$ μg in Hep G2-CR78 cells). The cytotoxicity of ara-M in infected cells paralleled the TK activity. Metabolic studies have demonstrated an increase of 7000-fold and 2000-fold of ara-A levels (compared to uninfected Hep G2 cells) in Hep G2-CR74 and Hep G2-CR78 infected cells.

This approach provided a model for HCC treatment. If for some reason normal hepatocytes do integrate the ALB TRS/VZV TK chimera and express TK activity, then conversion of the ara-M prodrug to ara-ATP will be non toxic to these normal hepatocytes which are non dividing as ara-ATP is cytotoxic only to dividing cells.

2.2. Purine nucleosides (acyclovir, 9-(2-hydroxy ethoxymethyl)guanine, ACV; ganciclovir, 9-[2-hydroxy-1(hydroxymethyl)ethoxy]methyl guanine, GCV; 1(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracyl, FIAU).

The system is based on the metabolic activation by the herpes simplex virus thymidine-kinase (HSV-TK) of certain purine nucleotides which have previously demonstrated clinical efficacy for the treatment of herpes in humans. HSV-TK catalyses the phosphorylation of the purine nucleoside analogues ACV, GCV and FIAU, that are poor substrates for the mammalian nucleoside monophosphate kinase. The conversion is to the corresponding nucleotide monophosphates which are catalysed to nucleotides triphosphates by the mammalian kinases. These latter nucleotides are able to inhibit DNA synthesis and thus kill dividing cells [23] (see Scheme 3). Evidence has been obtained for the inhibition by ACV of DNA polymerase, the elongation of the newly synthesised DNA and for its incorporation into DNA as a chain terminating event [23,24].

Early studies were undertaken in order to substantiate the 'tissue mosaicism' strategy [4,6]. This strategy assumes that genes which modify cellular sensitivity to various anticancer agents could be introduced prophylactically into a tissue so that some cells acquire a given gene while others do not. The result is a mosaic of cells that are different in sensitivity to the anticancer agent under consideration. It was hoped that if a tumour of clonal origin developed later from this tissue, it would acquire sensitivity to the particular prodrug while the host would be protected by those cells in the mosaic normal population which do not share this gene [4]. This strategy was tested using BALB/c murine cancer cells bearing the HSV-TK gene. Treatment with GCV in clonogenic assays showed that HSV-TK negative lines were resistant to doses 200–>1000 fold higher than HSV-TK positive lines. Treatment of TK^+-tumour bearing animals with GCV (150 mg/kg/injection, twice daily, for 5 days), after tumours became visible, induced complete regressions and long-term survivors. A bystander effect, called 'metabolic cooperation' was shown [4]. In contrast there were no survivors in the animals with TK^+ tumours that did not receive GCV or in TK^- tumour bearing animals treated with GCV or in animals treated with CP alone. Unfortunately, the



Scheme 3. Metabolism of acylclovir [23]. 1, human GMP-kinase; 2, nucleoside diphosphate kinases. The same metabolic pathway was reported also for GCV and FIAU [24].

mosaicism strategy suffers from the drawback that the gene transfection could not be confined to a single tissue and is therefore only justified for people with exceptionally high risk of malignancies (e.g. myelodysplastic syndrome) [6].

Further attempts were focused on generating curative models. A suitable vector for TK-gene transfection, plasmid pApS1, was engineered, which contained the TK gene under the control of the mouse mammary tumour virus long terminal repeat (MTV-LTR). This vector was transfected into rat 208 fibroblasts or Rat-2 clone 3B cells lines which subsequently became sensitive to ACV (60% cell death at 10 μM and 98% at 100 μM) or to FIAU (>60% cell death at 0.1 μM and 98% at 3.0 μM) [5]. The cytotoxicity was proportional to the levels of HSV-TK expression and to the concentrations of ACV or FIAU used.

Sarcoma (K2) and lymphoma (Ly18) HSV-TK$^+$ cell lines were obtained by transfection of the TK gene into the wild-type cell lines using NTK, STK and mTK retroviral vectors. A significant increase in GCV cytotoxicity ($IC_{50} < 0.2$ μg/ml) was observed in all transfected lines which expressed TK activity (K2, $IC_{50} = 0.005$ μg/ml; Ly18-NTK and Ly18-STK, $IC_{50} = 0.009$ μg/ml and Ly18-mTK, $IC_{50} = 0.007$ μg/ml). Mice with transplanted K2 sarcomas were sensitive to GCV treatment (140 mg/kg i.p. injection, 5 days after transplantation, twice a day for 5 days). Durable regressions were obtained in 12/19 animals [6]. In contrast, in lymphoma similar treatment induced only partial regressions, but the tumours regrew rapidly. This failure was attributed to genomic instability, since there was loss of HSV-TK activity.

Later more clinically relevant models have been developed. Brain tumours were the first choice for GDEPT [8,10–12,25] since neurones and other non-tumour cellular elements in the brain are non-dividing. Also vascular endothelial cells that respond to angiogenesis signals are present in the vicinity of the tumour, so their destruction is desirable. The blood-brain barrier is considered another advantage in this model. Clinically the model is useful since there is a poor prognosis of this type of tumour. It was demonstrated that established glioma C6 and 9L tumours in Fisher 344 rats could be cured when transfected with TK gene and subsequently treated with GCV. In dose-response assays both C6-TK and 9L-TK cells were 100 times more sensitive to killing with GCV than the parent wild-type lines. In long-term survival studies (90 days), all rats with HSV-TK transfected tumours that were treated with by GCV (50 mg/kg, once per day for 14 days) demonstrated improvements in survival (10/10 animals, while all controls died by day 25) [10].

In order to improve the low viral titer and the low target cell transduction frequency associated to retroviral vectors, the use of recombinant adenovirus vectors were suggested. Such a vector was constructed (pADL.1/RSV-TK, in which the TK-gene is controlled by the LTR-5′ of Rous sarcoma virus-RSV) to produce high levels of transduction in the C6 glioma cell line. On treatment with GCV (125 mg/kg, twice a day, 3 days followed by 100 mg/kg, twice a day, for another 3 days) of animals bearing C6 glioma, a 500-fold increase of tumour cell kill was obtained (or 2.7 log cell kill) compared to controls (average tumour volumes in treated mice: 0.054 mm^3 or 5.4×10^4 cells; compared to 28.2 mm^3 or 2.8×10^7 cells in non-treated animals). It was estimated that 50% of glioma cells were transfected [12].

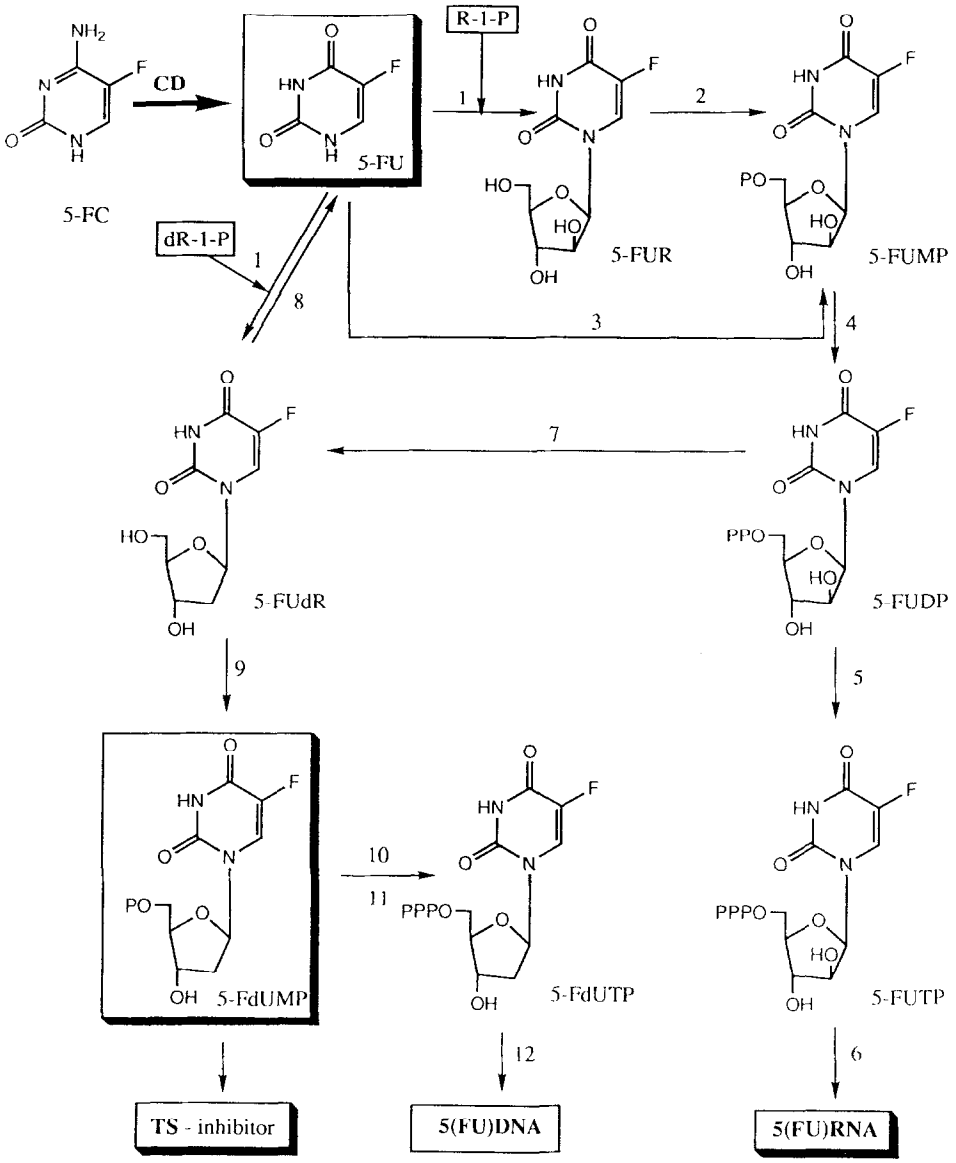
These studies demonstrated the feasibility of the transfection of the TK gene in vitro and in vivo and that significant therapeutic results with purine nucleoside prodrugs could be obtained. In addition other malignancies, e.g. melanoma [13,14,26], lung adenocarcinoma [27], breast carcinoma [28], head and neck squamous cells cancer [29] and liver metastasis [7]. They also showed that a bystander effect could be obtained in vivo.

2.3. 5-Fluorocytosine (5-FC)

CD is a bacterial enzyme (from *E. coli*) which is not present in human cells. This enzyme converts the non toxic 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to a potent pyrimidine antimetabolite. Its cytotoxicity depends upon cellular anabolism to 5-fluorodeoxyuridine-5′-monophosphate (5-FdUMP) and to 5-fluorouridine-5′-monophosphate (5-FUMP) by endogenous enzymes [30,31]. The latter is phosphorylated to 5-fluorouridine-5′-triphosphate (5-FUTP) which can result in the formation of fraudulent (5-FU)RNA. This can affect RNA maturation and processing and may sometimes be an essential event for cytotoxici-

ty. The former is a powerful inhibitor of thymidylate synthase (TS) through the formation of the covalently-linked ternary enzyme-inhibitor-cofactor complex resulting in an irreversible inhibition of this key enzyme for DNA biosynthesis and leading to cell kill (see Scheme 4) [30,31]. Another alternative is the conversion of 5-FdUMP by diphosphate kinases into the corresponding triphosphate (5-FdUTP), which is incorporated into the DNA, interfering with its biosynthesis [30,31].

In early reports, the CD gene was mutated to enhance its expression in eukaryotic systems [15]. In clonogenic assays, murine fibroblasts 3T3 cells transfected with CD gene were killed selectively by



Scheme 4. Metabolism of 5-FC [30,31]. 1, nucleoside phosphorylases; 2, uridine kinase; 3, phosphorybosyl-pyrophosphate dependent orotate phosphorybosyl transferase; 4, UMP-kinase; 5, nucleotide diphosphate kinases; 6, RNA polymerase; 7, ribonucleoside reductase; 8, thymidine and/or deoxy-uridine phosphorylases; 9, deoxyuridine kinase; 10, dUMP-kinase; 11, (deoxy)nucleoside kinases; 12, DNA polymerase. R-1-P, ribose-1-pyrophosphate; dR-1-P, deoxyribose-1-pyrophosphate.

incubation with 5-FC (65 μg/ml) whereas control untransfected cell lines were not. Assays of mixtures of transfected with untransfected cells showed that CD^+ cells were killed without any bystander effect [15].

CD transfections of non metastatic colon adenocarcinoma 38 cells, and non-metastatic colon 102 and 205 methylcholanthrene-induced fibrosarcoma cells with vector containing the CD and NeoR genes were performed. There was a clear dose-response relationship between 5-FC concentration and killing in the transfected cell lines [16]. Mice injected with 1×10^5 cells from the three lines all developed tumours. Treatment was started 3–5 days later and 5-FC (37.5 mg/kg) was administered twice a day for 10 days. The experiment was followed for 8 weeks. Tumour incidence was 6% in the 102 CD colon fibrosarcoma and 38 CD colon adenocarcinoma tumour bearing animals and 47% in 205 CD colon fibrosarcoma. In all controls the incidence was 100%. It was noticed that the animals whose CD^+ tumours were eliminated in vivo by treatment with 5-FC were resistant to subsequent rechallenge with unmodified wild-type tumour indicating an immune response [16].

Studies were carried out to develop a therapeutic model for colorectal carcinoma using the CD/5-FC system. In the first step, it was demonstrated that significant antitumour effects could be obtained in vivo with 5-FC treatment when tumour colorectal cells were transfected with the CD gene [17]. The human colorectal tumour cell line, WiDr, was transfected with the plasmid pCMV/CD-1 which contains the CD gene. The prodrug/drug ratio was approximately 5000 in WiDr cells (5-FC, IC_{50} 26 000 μM; 5-FU, $IC_{50} = 5$ μM). In WiDr-CD cells the IC_{50} of 5-FC shifted to 27 μM. No effect on the growth rate or sensitivity to 5-FU was observed in transfected cells. Data obtained in animals showed that: (a) 100% tumour regressions and some tumour cures could be obtained without systemic toxicity using short courses of treatment with 5-FC (500 mg/kg i.p. for 10 days); (b) by increasing the dosing period an approximate 75% cure rate could be obtained; (c) intraperitoneally administration was better than an intravenous infusion [17].

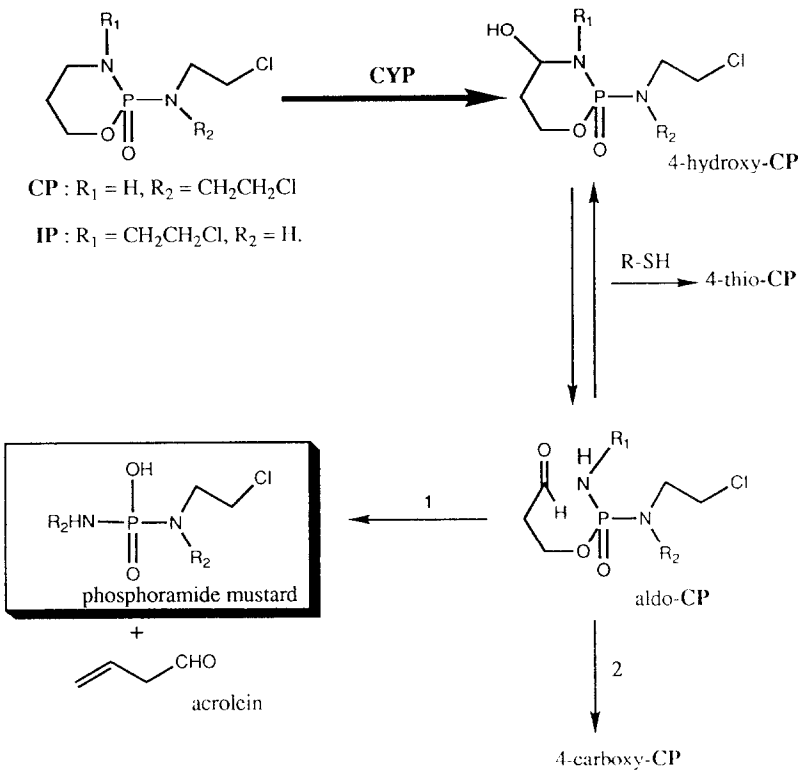
It was demonstrated that only a small percentage of the cells within the solid tumour were required to express CD in order to generate a significant antitumour effect with 5-FC treatment [2]. Tumour xenografts containing CD expressing cells could generate concentrations greater than 400 μM 5-FU within the tumour. This was also liberated into the surrounding environment and CD^- cells were killed in vitro and in vivo indicating a bystander effect. In mixing experiments when only 2% of the tumour cells were CD^+, significant regressions were observed after treatment with non-toxic doses of 5-FC (500 mg/kg i.p.) [2].

2.4. Cyclophosphamide (CP) and isophosphamide (IP)

The use of CP and IP were considered for GDEPT since they are activatable prodrugs and also detailed biochemical and pharmacological studies were available for these compounds. It was known that both CP and IP are activated in rat liver by microsomal mixed-function oxidases cytochrome-P450 (CYP) to 4-hydroxy-CP, which exists in equilibrium with its ring opened tautomer, aldophosphamide as shown in Scheme 5 [32].

This reaction requires NADH and O_2 and its $K_m = 0.5–1.5$ mM [32]. There is evidence that the significant circulating and pharmacologically important metabolite is 4-hydroxy-CP/aldo-CP. The cytotoxic metabolite is the corresponding phosphoramidic mustard which results together with acrolein from the decomposition of aldo-CP (see Scheme 5). The spontaneous β-elimination of acrolein from aldo-CP is a base catalysed process (involving OH^-, carbonate anion and organic or inorganic phosphate) [32]. There are however data suggesting that 3′,5′-exonucleases (and especially those related to DNA polymerase) catalyse the release of the phosphoramide mustard and this could be the basis of its relative specificity to the cancer cells [33]. The activation of IP occurs in a similar way [34]. These bifunctional alkylating agents kill both cycling and non-cycling cells by cross-linking DNA.

Cytochrome P-450 represents a very complex enzymatic system involving many isoenzymes with quite different substrate specificity. Not all the isoenzyme are equally effective in activating CP or IP. Isoenzymes 2B1 (CYP2B1, phenobarbital-inducible), 2C6 (CYP2C6) and 2C11 (CYP2C11) (both constitutively expressed) are the enzymes involved in activating oxazaphosphorines in rats. The iso-



Scheme 5. Metabolism of cyclophosphamide and isophosphamide [32,34]. 1, 3′,5′-exonucleases; 2, aldehyde oxidase. R-SH, endogenous SH-compounds.

enzymes 2B6 (CYP2B6) and 3A4 (CYP3A4) are responsible for this process in human liver [35]. These enzymes are present only in liver and not in other normal tissues or in tumour cells, which was the rationale in trying to generate CYP2B1 activity in cancer cells.

A study was undertaken in order to assess whether transfection of the CYP2B1 gene in glioma 9L cells followed by CP or IP treatment would represent a useful GDEPT model for further clinical developments [20]. The 9L cells were transfected with a rat CYP2B1 expression plasmid and 9L-ZL and L450-2 cells lines sensitive to CP ($IC_{50} = 70$ μM) or IP ($IC_{50} = 145$ μM) were obtained. In contrast, the parental 9L control line showed no cytotoxic effects when grown in the presence of millimolar concentrations of CP or IP. The difference in the IC_{50} figures of CP and IP is in good agreement with the kinetic data, which showed that the CYB2B1 enzyme cleaves IP with an efficiency (V_{max}/K_m) 3–4-fold lower than CP [20]. Rats were injected with parental or transfected tumour cells (9L-ZL, L450-2) (2×10^6 subcutaneously). Treatment with a single injection (i.p.) of CP (100 mg/kg) was started 7 days after transplantation. Complete tumour growth inhibition and long term survivors (7–8 weeks) were obtained for the 9L-ZL cell line (8/9 animals) and the L450-2 cell line (11/11 animals). A bystander effect accounts for these results. In contrast no tumour inhibition was obtained with the parental control line (0/11 animals).

An important finding was that in the transfected cell lines CP treatment was highly effective without an apparent increase in host toxicity. This is especially important since significant levels of CYP, which is capable of activating CP, is present in normal liver. However, since in liver the activating enzyme is CYP2C6 as distinct from the CYP2B1

which is used in this therapy, the former could be inactivated by CYP-isoform inhibitors, e.g. 21,21-dichloro-progesterone or 3,5-dimethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine [36,37].

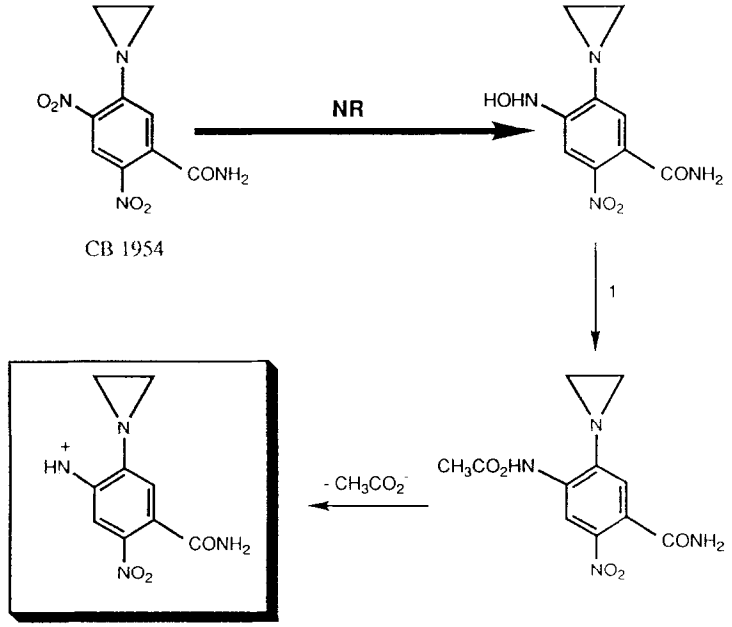
2.5. 5-Aziridinyl-2,4-dinitrobenzamide (CB 1954) activated by nitroreductase (NR)

In the 1960s, CB 1954, a monofunctional alkylating agent, was found to be extremely potent against the Walker carcinosarcoma in rats but inactive against a panel of tumours used in screening anticancer agents [38]. It was found that this was due to its specific activation by the enzyme DT-diaphorase which is present in this rat tumour and which can convert the compound into the effective bifunctional alkylator: 5-aziridinyl-4-hydroxylamino-2-nitrobenzamide [39,40]. This is probably further converted by acetyl CoA or other endogenous enzymes to a more potent electrophile, for example, the corresponding acetate which further reacts with DNA. The metabolism of CB 1954 is shown in Scheme 6. Since the active drug is an alkylating agent it will kill both cycling and non cycling cells, which is an advantage compared to other GDEPT systems (e.g. GCV/TK or 5-FC/CD). The human DT-diaphorase is much less able to activate CB 1954 than is the rat enzyme.

It has subsequently been found that the bacterial nitroreductase (NR) is also much more effective than the human DT-diaphorase in activating the prodrug [41]. A recent study describes the transfection of the NR (*E. coli*) gene in NIH3T3 cells, using a recombinant retrovirus. The NIH3T3 cells expressing NR were killed after CB 1954 administration. Co-operative killing was observed when cells expressing both NR and TK were treated with a combination of CB 1954 and GCV, supporting their different mechanism of action. Human melanoma, ovarian carcinoma and mesothelioma cells all became sensitive to CB 1954 after transfection of the NR gene [21].

3. The bystander effect

The 'bystander effect' can be defined as the tumour regression which take place after prodrug administration when only a fraction of the tumour mass is genetically modified with foreign enzyme [42]. The striking successes described in GDEPT would surely not be possible without the existence of



Scheme 6. Metabolism of CB 1954 [39]. 1, acetyl-coenzyme A.

such an effect. As mentioned earlier, in some models only 2% of genetically modified cells are enough to obtain therapeutically significant results [2].

The mechanism responsible for this effect is not well understood. Several explanations are plausible and there is the possibility that more than one mechanism is involved. On the other hand it is also possible that the mechanisms are different when different types of prodrugs are involved. The main possibilities are briefly discussed:

1. Toxic metabolites are formed during prodrug activation from dead and dying cells. This is postulated for 5-FU (formed from 5-FC) and for the metabolites (aldophosphamide, phosphoramidic mustards or acrolein) of CP or IP [2,20]. This assumption is supported by the fact that no cell-to-cell contact is required in order to obtain a bystander effect in these model systems. In WiDr cells, this effect was observed both in vitro and in vivo and 30% of cells expressing CD is enough to eradicate the whole cell population [2]. This bystander effect is more significant in vivo than in vitro even when athymic mice are used indicating that the immune response is not involved. This explanation does not apply to purine nucleosides where the toxic metabolites, being phosphorylated, are not diffusible across cell membranes.
2. In the case of purine nucleosides, a direct cell-to-cell contact is required in order to achieve a bystander effect [42]. When TK^+ cells are separated by a 0.4 mm filter membrane from TK^- cells, the bystander effect is abrogated. Because the toxic metabolites formed during purine nucleosides activation cannot cross cells membranes, it was postulated that this event could occur through apoptotic vesicle transfer from TK^+ cells to TK^- cells. There is evidence that vesicle transfer takes place [42].
3. It was argued also that the gap junctions are involved in the transfer of toxic metabolites from cell to cell [42,43].
4. It is also possible that the TK is transported by apoptotic vesicles or through gap junctions [44].
5. The transfer of hydrolases or other lytic enzymes triggered by programmed cell death from TK^+ to TK^- cells was also suggested; [42]
6. Killing of tumour cells by apoptosis could heighten the immune response to wild type tumour cells by a priming effect [42].

4. The design of prodrugs for GDEPT

The design of prodrugs for GDEPT should address several questions related to clinical situations. Some of these are:

1. penetration of prodrugs into cancer cells;
2. low toxicity of prodrugs;
3. effective activation of prodrug by the expressed enzyme/favourable activation kinetics;
4. high cytotoxicity of the active drug;
5. ideally the released drugs should be effective in both cycling and non-cycling cells;
6. the released drugs should induce a bystander effect.

For GDEPT lipophilic prodrugs are required in order to penetrate across cell membranes or prodrugs could be synthesised that are taken up by active transport. It is believed that the prodrugs so far in use (nucleoside analogues, 5-FC, CP, CB 1954) are taken up by cells by passive diffusion.

Low cytotoxicity for the prodrugs is essential since they will taken up into normal cells. The activation of the prodrugs is a critical step in GDEPT. Possibly, it is desirable that the prodrug activation to the active drug should proceed directly by the expressed enzyme, without the requirement for further catalysis since it is possible for the endogenous enzymes to be defective in cancer cells which could lead to an incomplete conversion to the active drug. This is possible for 6-methoxy purine arabinonucleoside, GCV and ACV where the pathway to the toxic metabolite after the activation by bacterial expressed TK, involves mammalian adenosine monophosphate deaminase AMP- and/or GMP-kinases (see Schemes 2 and 3). This is also the case of 5-FC which is converted to 5-FU by CD. The active metabolite is however the 2′-deoxy-5-fluorouridine-5′-monophosphate (5FdUMP) or 5-FUTP which results from 5-FU conversion by a number of mammalian enzymes involving a complicated activation pattern (see Scheme 4). This sophisticated activation pathways of these antimetabolites is par-

tially responsible for their propensity to induce resistance.

The alkylating agents present, from this point of view, an advantage compared to many other anticancer compounds, since the active moiety is directly released after the prodrug cleavage by the expressed enzyme. With CP and CB 1954 as prodrugs the activation is much less dependent on endogenous enzymes than the previously discussed prodrugs. CP after the primary activation by CYP, requires a second step by 3′,5′-exonuclease to generate the active metabolite. For CB 1954, the 5-aziridinyl-4-hydroxylamino-2-nitrobenzamide intermediate resulting from the prodrug reduction by NR, needs a further enzymatic step to be converted in the powerful electrophile which alkylates DNA.

High cytotoxicity and optimum half-lives are required from the drugs in order to maximise the effect. The half-lives should be short enough not to allow the drug to leak out from the tumour and long enough to induce the bystander effect. Good kinetic parameters (higher k_{cat}) seem to favour the activity. This effect was noticed when the activity of CP (higher k_{cat}, lower IC_{50}) was compared with that of IP (lower k_{cat}, higher IC_{50}) [20].

Ideally the released drug should be effective against both cycling and non-cycling cells. Unfortunately the prodrugs so far used in GDEPT are mainly antimetabolites, which require cycling cells (S phase) for cytotoxicity. It has been speculated that the resistance to GCV in GDEPT is not an acquired one but resulted from tumour cells being in G_0 during the time of GCV administration [45]. This argument is supported by the fact that the tumours which grew out remained sensitive to GCV. The use of prodrugs which, after activation release alkylating agents, should have a potential advantage over purine nucleosides or 5-FC, being cytotoxic also against non-cycling cells. No data were available for CP and IP, but it was shown that NR/CB 1954 system was effective in non-cycling cells [21]. Alkylating agents also have the advantage that they are dose dependent and less prone than other anticancer compounds to induce resistance [46,47].

Another important aspect is that the released drugs should be able to induce the 'bystander effect'. All systems already reported exhibit a significant bystander effect, at least in vivo. However the mechanism may be different for different types of drugs. For instance for the toxic nucleoside phosphates resulting from the activation of purine nucleosides (GCV, ACV, etc.) and which are non-diffusible across cell membranes, a cell-to-cell contact is required to obtain a bystander effect [42]. In contrast for diffusible drugs like 5-FU or aldophosphamide (or phosphoramide mustard) no such requirement is necessary [2,20]. A bystander effect was shown also for CB 1954 [21].

5. Conclusions

The following steps have been achieved:

1. tumour cells after transfection in vitro with the gene for an enzyme, have been shown to activate specific prodrugs, converting them to cytotoxic drugs [18];
2. transfected tumour cells, when injected into animals, are able to grow and to generate tumours, which have subsequently been cured by administration of appropriate prodrugs;
3. the 'bystander effect' has been observed both in vitro and in vivo. A bystander effect occurs when not only the transfected tumour cells, but also wild-type tumour cells, are killed after administration of the prodrugs which are cleaved to active drugs capable of diffusing or being actively taken up into cells not expressing the foreign enzyme [42];
4. gene transfection has been achieved in vivo using direct DNA injection (naked DNA) or gene infection has occured with retroviral vectors which target DNA-synthesizing cells;
5. gene infection has been achieved in vivo using cells (packaging cells) continuously producing the desired retroviral vector [9].

The demonstration of steps 1–5 in addition to the prodrugs described herein combine to illustrate both the feasibility of the currents systems and the future promise for GDEPT.

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