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Prodrugs in Cancer Chemotherapy

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At present, chemotherapy is not very effective against common solid cancers especially once they have metastasised. However, laboratory experiments and studies on dose intensification in humans have indicated that some anti-cancer agents might be curative but only if the dose given was very much higher than that presently obtainable clinically. Prodrugs, activated by enzymes expressed at raised level in tumors, can deliver at least 50-fold the normal dose and can cure animals with tumors normally resistant to chemotherapy. This approach has not yet proved to be practicable clinically because of the rarity of human tumors expressing a high level of an activating enzyme. However, new therapies have been proposed overcome this limitation of prodrug therapy. Enzymes that activate prodrugs can be directed to human tumor xenografts by conjugating them to tumor associated antibodies. After allowing for the conjugate to clear from the blood a prodrug is administered which is normally inert but which is activated by the enzyme delivered to the tumor. This procedure is referred to as ADEPT (antibody-directed enzyme prodrug therapy). Early clinical trials are promising and indicate that ADEPT may become an effective treatment for all solid cancers for which tumor associated or tumor specific antibodies are known. Tumors have also been targeted with the genes encoding for a prodrug activating enzymes. This approach has been called gene-directed enzyme prodrug therapy (GDEPT) or VDEPT (virus-directed enzyme prodrug therapy) and has shown good results in animal models. These new therapies may finally realise the potential of prodrugs in cancer chemotherapy. (Pathology Oncology Research Vol 3, No 4, 309–324, 1997)

Key words: antibody targeting, drug targeting, gene therapy, antitumor agents

Introduction

The majority of the agents that are now used in cancer chemotherapy act by an anti-proliferative mechanism. Therefore, their action is on cells that are in cycle, or in some cases, on a specific phase of the cell cycle rather than by a specific toxicity directed towards a particular type of cancer cell. Predictably, the limiting toxicity of the majority of anti-cancer agents is thus a result of a toxic effect on the normal host tissues that are the most rapidly dividing such as bone marrow, gut mucosa and the lym-

phatic system. Further, most human solid cancers do not have a high proportion of cells that are rapidly proliferating and they are therefore not particularly sensitive to this class of agent. The result is that, because of host toxicity, treatment has to be discontinued at dose levels that are well below the dose those that would be required to kill all viable tumor stem cells. It has been calculated for the more resistant solid cancers, that to achieve cure, total doses of more than a hundred times the normal clinical dose of present day agents would need to be given. The poor selectivity of anti-cancer agents has been recognised for a long time and attempts to improve selectivity and allow greater doses to be administered have been numerous. Early on, different classes of chemical with anti-proliferative activity but which differed in their normal tissue toxicity, were put together in drug combinations thus improving the total dose of cytotoxic agent administered. Subsequently, clinical trials have taken place of chemicals
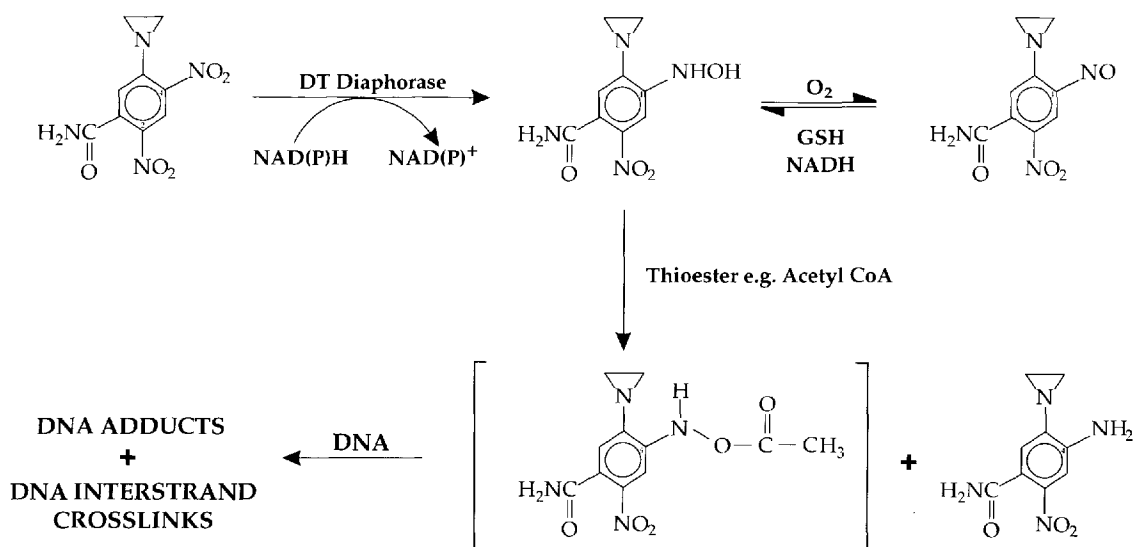
Figure 1. The bioactivation of CB 1954. The initial step is the reduction of CB 1954 by the enzyme DT diaphorase to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This hydroxylamine derivative can react with thioesters to produce a DNA reactive species. It is postulated that this is the N-acetoxy derivative. A bacterial nitroreductase can also perform this reduction but produces a mixture of the 2- and 4-hydroxylamines.

that either sensitise the cancer to the administered chemotherapy or protect the sensitive host tissues. A protein and a pentapeptide that protect normal stem and early progenitor cells probably by altering their proliferation rate will soon be on clinical trial. Biological agents such as GCSF (granulocyte colony stimulating factor), which stimulate the recovery of bone marrow following cytotoxic therapy and autologous bone marrow replacement after treatment, have allowed the administration of higher than normal clinical doses of certain anti-cancer agents. The local application of drugs by, for example by injection into the hepatic artery for the treatment of liver metastases or limb perfusion for melanoma, also allows higher doses of cytotoxic drugs to be given. However, it is nowhere near the hundred-fold increase required for the complete regression of resistant solid cancers.

Prodrug therapy

At one time it was thought that a sufficiently high degree of selectivity might be obtained by the use of prodrugs. Prodrugs are defined as chemicals that are toxicologically and pharmacodynamically inert but which may be converted in vivo to active products. Conversion of the prodrug to the active form can take place by a number of mechanisms depending, for example, on changes of either pH, oxygen tension, temperature, salt concentration or by spontaneous decomposition of the drug or internal ring opening or cyclisation. A major approach in prodrug design is the synthesis of inert analogues that are converted to the active drug by enzyme action. In cancer chemotherapy, the prodrug would

be inert but converted in vivo into a highly toxic metabolite by an enzyme present in the cancer cells but not in other cells (Table 1). An example of the high degree of selectivity that can be attained with prodrugs is shown in Figs 1 and 2. The di-nitro compound CB 1954 is a prodrug containing an aziridine ring. CB 1954 is only a weak mono-functional alkylating agent (by virtue of the aziridine function) and is not highly cytotoxic. However, in the presence of the enzyme DT-diaphorase the 4-nitro group is reduced to the hydroxylamino that is then rapidly acylated to a bifunctional alkylating agent (Figure 1).[1,2] There is a least a 10,000 fold difference in toxicity between the Walker cell line that has a high level of the activating enzyme and Chinese hamster V79 cells that lack the enzyme (Figure 2).[2,3] Thus, CB 1954 can actually cure the Walker tumor when implanted into either rats[4] or mice.[5] In another example in a tumor bearing animal, a relatively non-toxic alkylating agent is activated by the enzyme β-glucuronidase to an extremely reactive and toxic metabolite[6] (Figure 3). The degree of binding to the DNA target site is also much greater than that achieved by an equivalent dose of a similar alkylating agent that is not activated in vivo.[7] Thus, it would thus appear that the appropriate use of prodrugs could deliver much higher doses of drug than normal and might therefore be effective against common solid cancers.

Prodrugs have been made to exploit many situations where it is thought that cancers produce unique enzyme profiles. For example, it has been claimed that a characteristic feature of many cancers is the overproduction of a serum plasminogen activator. This enzyme hydrolyses plasminogen to the active protease plasmin that may play a

Table 1. Enzymes and prodrugs that have been proposed for cancer therapy. The enzymes may have been targets for prodrug therapy or targeted using ADEPT or GDEPT.

| Enzyme	Prodrug	Drug	Application
Carboxypeptidase G2	Benzoic acid mustard glutamates	Benzoic acid mustards (various)	ADEPT (GDEPT)
Thymidine kinase (viral)	Ganciclovir Adenine arabinonucleoside (araM)	Ganciclovir triphosphate Adenine arabinonucleoside triphosphate (araATP).	GDEPT
Glucose oxidase	Glucose	Hydrogen peroxide	Radical generating system
Xanthine oxidase	Hypoxanthine	Superoxide, hydrogen peroxide	Radical generating system
Cytosine deaminase	5-fluorocytosine*	5-fluorouracil	GDEPT ADEPT
Carboxypeptidase A/B	Methotrexate-alanine α-linked derivatives of TS inhibitors	Methotrexate TS inhibitors	ADEPT
α-Galactosidase	N-[4-(a-D-galactopyranosyl)-benyloxycarbonyl]-daunorubicin	Daunorubicin	ADEPT
β-Glucosidase	Amygdalin	Cyanide	ADEPT
Plasmin	Peptidyl-p-phenylenediamine-mustard	Phenylenediamine-mustard	Prodrug Therapy
Azoreductase	Azobenzene mustards*	Phenylenediamine-mustards (various)	Prodrug Therapy
γ-Glutamyl transferase	γ-Glutamyl p-phenylenediamine-mustard	Phenylenediamine-mustard	Prodrug Therapy
β-Glucuronidase	Phenolmustard-glucuronide Epirubicin-glucuronide	Phenolmustard Epirubicin	Prodrug Therapy ADEPT
β-Lactamase	Vinca-cephalosporin Phenylenediamine mustard-cephalosporin Nitrogen-mustard-cephalosporin	4-Desacetylvinblastine-3-carboxhydrazide Phenylenediamine mustard Nitrogen mustards (various)	ADEPT
Alkaline phosphatase	Phenolmustard phosphate Doxorubicin phosphate Mitomycin phosphate Etoposide phosphate	Phenolmustard Doxorubicin Mitomycin alcohol Etoposide	ADEPT
Penicillin amidase	Palytoxin-4-hydroxyphenyl-acetamide Doxorubicin-phenoxyacetamide Melphalan-phenoxyacetamide	Palytoxin Doxorubicin Melphalan	ADEPT
Nitroreductase	5-(Aziridine-1-yl)-2,4-dinitro-benzamide (CB 1954) 4-Nitrobenzyloxycarbonyl derivatives	5-(Aziridine-1-yl)-4--hydroxylamino-2-nitrobenzamide e.g. Actinomycin D, mitomycin C	ADEPT GDEPT
DT-diaphorase	CB 1954*	5-(aziridine-1-yl)-4-hydroxylamino-2-nitrobenzamide	Prodrug Therapy
Aminopeptidase	2-L-Pyroglutamyl-methotrexate	Methotrexate	Prodrug Therapy ADEPT
Thymidine phosphorylase	5'-Deoxy-5-fluorouridine () 5'-DFUR	5-FU	GDEPT
Deoxycytidine kinase	Ara-C	Adenine arabinonucleoside triphosphate (araATP)	GDEPT
Cytochrome-P450	Cyclophosphamide Ifosfamide	Phosphamide mustard (+ acrolein?)	GDEPT
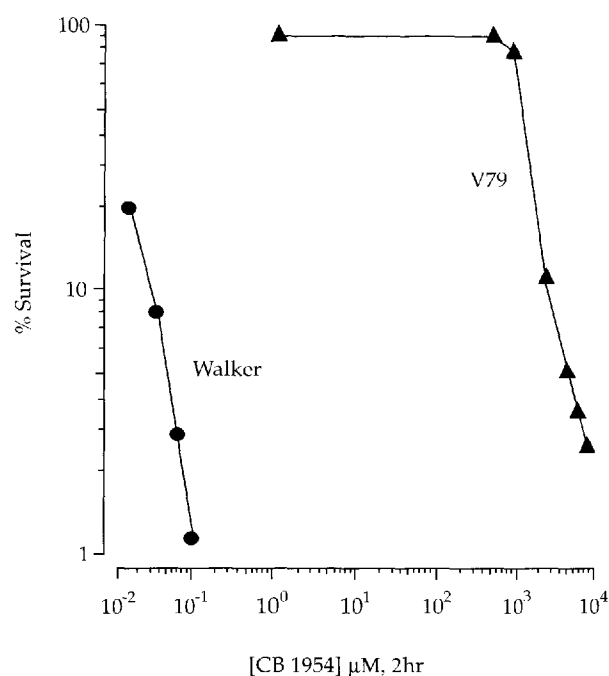
Figure 2. The effect of CB 1954 on the colony forming ability of Walker 256 cells (which contain high levels of DT diaphorase) or Chinese hamster V79 cells (very low levels of DT diaphorase). All treatments were for 2 hours at 37°C. The concentration of CB 1954 is plotted on a logarithmic scale.

key role in the spread of tumors. Chicken embryo fibroblasts have a low level of plasminogen activator but if they were transformed by the Rous sarcoma virus there was a dramatic rise in the enzyme.[8] This has been exploited by designing the prodrug shown in Figure 4.[8] On hydrolysis at the specifying sequence – LEU-LYS – the highly cytotoxic p-phenylenediamine mustard was generated. This mustard has a short chemical half-life that may confine its cytotoxic effects to the tumor environment in which it has been generated. In another example, a γ-glutamyl derivative of p-phenylenediamine mustard was synthesised when it was shown that malignant hepatocytes over expressed γ-glutamyl transferase.[9] This enzyme is located on the cell surface and transfers the prodrug into the cell, removing the γ-glutamyl group in the process. Cells that do not have the enzyme are insensitive to the prodrug, partly because they do not internalise it and partly because the prodrug is a less chemically reactive than p-phenylenediamine mustard (and therefore less cytotoxic). Thus, the prodrug is specifically toxic for malignant cells that express the enzyme. Many cytotoxic agents can be converted into their γ-glutamyl derivatives and potentially used in combination.

Prodrugs have been shown to be very selective in animal models when an activating enzyme is either uniquely or over expressed in experimental tumors. However, clinical studies have been disappointing.[2] For example, in the

case of CB 1954 it has been shown that this prodrug is a much poorer substrate for the human form of the enzyme DT diaphorase than it is for the rat form.[2,10] Thus it does not show the marker anti-tumor effect against human tumors that were demonstrated in rat tumors. An aniline mustard prodrug activated by β-glucuronidase was also subject to a preliminary clinical trail. A correlation was shown between glucuronidase activity and patient response, but the occurrence of high β-glucuronidase activity was rare and not predictable by tumor type. It was not considered to be a feasible therapy.[11]

Antibody-Directed Enzyme Prodrug Therapy (ADEPT)

One of the approaches to overcome the intrinsic problems associated with the administration of cytotoxic drugs was drug targeting. This came about with the advent of monoclonal antibody technology in the 1970s was thought at the time to herald the fruition of the search for the "magic bullet" first proposed by Ehrlich in 1906.[12] It involves the conjugation of a cytotoxic agent to an antibody or other molecules that can specifically bind to antigen or receptor sites that are over expressed on the target tumor cell. However, the goal has proved to be elusive and a number of limitations are apparent in the use of this approach for the treatment of solid tumors in humans. These include: poor penetration of the tumor, lack of inter-tumor accumulation of the cytotoxic component and the heterogeneity of tumor associated antigens.[13-15] However, Bagshawe[16,17] suggested how the disappointing clinical results seen with drug targeting could be overcome. Activating enzymes, not drugs, could be targeted to human tumors using tumor associated monoclonal antibodies prior to administration of a prodrug. This approach has been termed ADEPT (antibody-directed enzyme prodrug
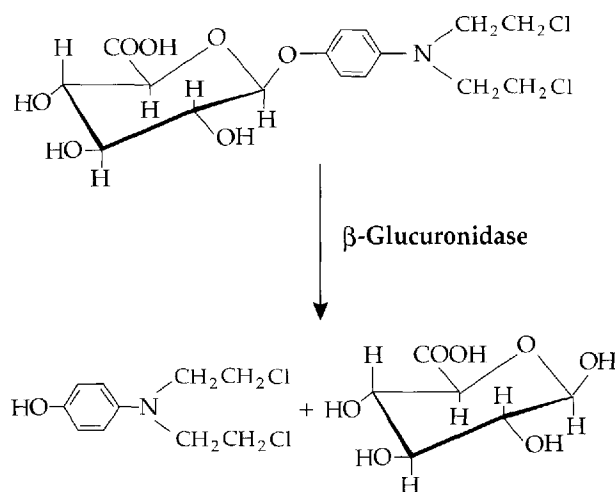

Figure 3. The formation of aniline mustard from its glucuronide by the enzyme β-glucuronidase.

therapy). For ADEPT, an enzyme of non-human or non-mammalian origin can be used and this could metabolise substrates not normally activated in humans. The enzyme is linked to a tumor associated antibody (this can be done either chemically or by using recombinant DNA techniques) and allowed to localise to the tumor.[16-19] Thus, ADEPT creates a tumor environment with a high concentration of an enzyme that would convert a normally inert substrate to a highly reactive metabolite *(Figure 5)*. The unlocalised conjugate is either given time to be eliminated from the body, or is hastened on its way by a "clearance" antibody. A non-toxic prodrug is then administered and activated at the site of the tumor by the bound conjugate *(Figure 5)*. The active drug can be of low molecular weight and can therefore rapidly diffuse and reach tumor regions not accessible to the antibody-enzyme conjugate[16-19] *(Figure 5)*. The targeted antibody-enzyme conjugate remains extra-cellular. It should not be internalised because the conjugate would be expected to be rapidly degraded in the lysosomal compartment. Further, the prodrugs can exploit the external enzyme by being charged and thus excluded from the cell until activated. Secreted antigen, if it accumulates within the interstitial spaces of the tumor, can also be exploited to achieve higher levels of the conjugate at the tumor. A major advantage of the ADEPT approach (over, for example, drug targeting or immunotoxins) is that it is catalytic and a single enzyme molecule can, in theory, generate many hundreds of active molecules per second from the prodrug. Further, because, the enzyme-conjugate is, by itself, inactive; it can be allowed to clear before administration of the prodrug. Not all tumor cells would be required to bind the antibody since the drug released by the enzyme could diffuse to neighbouring cells not expressing the antigen. It is fundamental to the concept of ADEPT that the prodrug is not activated by normal human enzymes. The enzyme must

Figure 4. The activation of a peptidyl prodrug by plasmin to form p-phenylenediamine mustard.

therefore have little equivalent activity in humans particularly in serum as the prodrug may be excluded from entering cells. Further, the enzyme must be active under physiological conditions and remain active when conjugated to an antibody.

A number of enzymes and prodrugs have that have been considered for ADEPT. They have been reviewed in detail,[20-22] are summarised in *Table 1*, and will not be discussed in detail. These include carboxypeptidase G2 *(Figure 6)*, alkaline phosphatase, ß-lactamase, penicillin amidase and cytosine deaminase. Mention should also be made of glucose oxidase that was the first example of an antibody-targeted enzyme[23] and was made before the advent of monoclonal antibody technology. The enzyme generates hydrogen peroxide upon oxidation of glucose. However, this system was not very cytotoxic and glucose is a poor choice of prodrug for ADEPT because it is normally present in serum.

Experimentally, ADEPT has been extraordinarily successful and has caused regression of many different types of human tumor xenografts many of which are resistant to standard therapy *(Figure 7)*. These experimental studies have been followed by a limited clinical study of ADEPT in patients being treated for advanced metastatic colon or rectal cancer and all had previously received extensive chemotherapy.[24,25] The trial used a conjugate of an antibody directed to the human carcinoembryonic (CEA) antigen linked chemically to a bacterial enzyme, carboxypeptidase G2, which can hydrolyse folates and glutamate prodrugs. The prodrug used was 4-[N-(2-chloroethyl)-N-(2-mesyloethyl)amino]-benzoyl-L-glutamic acid and this was well tolerated by patients (doses 200-2500 mg/m2 given as either 6 or 12 doses divided over 3 days). Four patients relieved 20,000 units/m2 of an anti-CEA F(ab')2-carboxypeptidase G2 conjugate. After 2 days, a clearing antibody was administered and the prodrug given when serum carboxypeptidase G2 levels were <0.02 units/mL. Circulating levels of CEA and an other tumor marker (19-9) fell in all four patients by 10-15 days after therapy. In two cases there was a measurable decrease in the size of liver metastases and in another case the patient became free of jaundice but with no decrease in the size of the metastases. Subjective responses, such as weight gain, lessening of pain and generally improved health were also reported. However, adverse effects were also reported. After 10-12 days an antibody response was raised against both the antibody and enzyme components of the conjugate.[24,25] Thus, the treatment is effectively limited to a single round of therapy although it was proposed to prevent this antibody response by use of cyclosporin A to immunosuppress the patient. The two patients who received conjugate and the highest dose of prodrug developed myelosuppresion. Presumably this is due to the active drug being released into the circulation and being
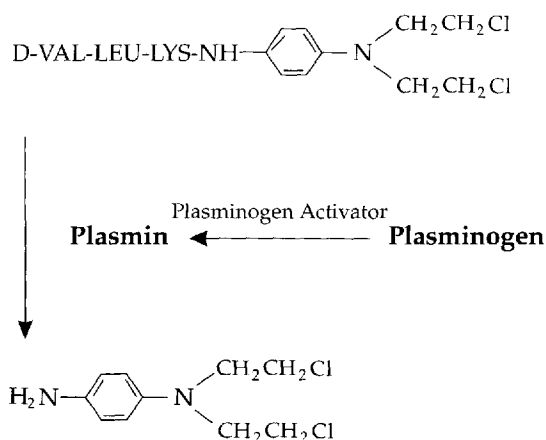
able to migrate to the bone-marrow. The active drug could be detected[24-26] in serum samples and this suggests that the release of a more reactive drug would be beneficial. With the demonstration that carbamates were also reasonable substrates for CPG2, the way was open to make a series of derivatives that would be converted by the enzyme to highly toxic and reactive chemicals that would probably hydrolyse before reaching sensitive host tissues[27] *(Figure 6)*. It is planned that chemicals of this structure will shortly be on clinical trial.

Problems associated with ADEPT include, the immunogenicity of the conjugate; activation of the prodrug by, unbound, circulating conjugate; conjugate heterogeneity and optimisation of the pharmacology of the active drug.[20-22] Many of the problems are being addressed using recombinant DNA technology. It has already been possible to obtain highly specific tumor associated antibodies using filamentous phage display.[28,29] Further, there is every possibility that fusion proteins will

be constructed between enzymes and antibodies that have been engineered to be non antigenic allowing several cycles of ADEPT therapy to be given.[30,31] The ultimate refinement, which is now possible by protein engineering techniques, is to make a bifunctional antibody in which one arm possesses an enzyme-mimicking catalytic function. Indeed, a catalytic antibody (termed an "abzyme") has been made which, *in vitro*, can produce cytotoxicity.[32] The abzyme can hydrolyze the carbamate prodrug 4-[N,N-bis(2-chloroethyl)]aminophenyl-N-[(1S)-(1,3-dicarboxy) propyl]carbamate to generate the corresponding nitrogen mustard. With so many prodrugs now already shown to be effective one might envisage combination therapy using different enzyme, antibody and prodrug combinations that might overcome any problems arising from tumor heterogeneity. Thus there is optimism that ADEPT, in some form or another, will eventually be used to treat the common solid cancers that are now refractory to chemotherapy.

Antibody-enzyme conjugate binds to tumor cells

Unbound antibody is cleared

Bound enzyme converts administered prodrug to its active form

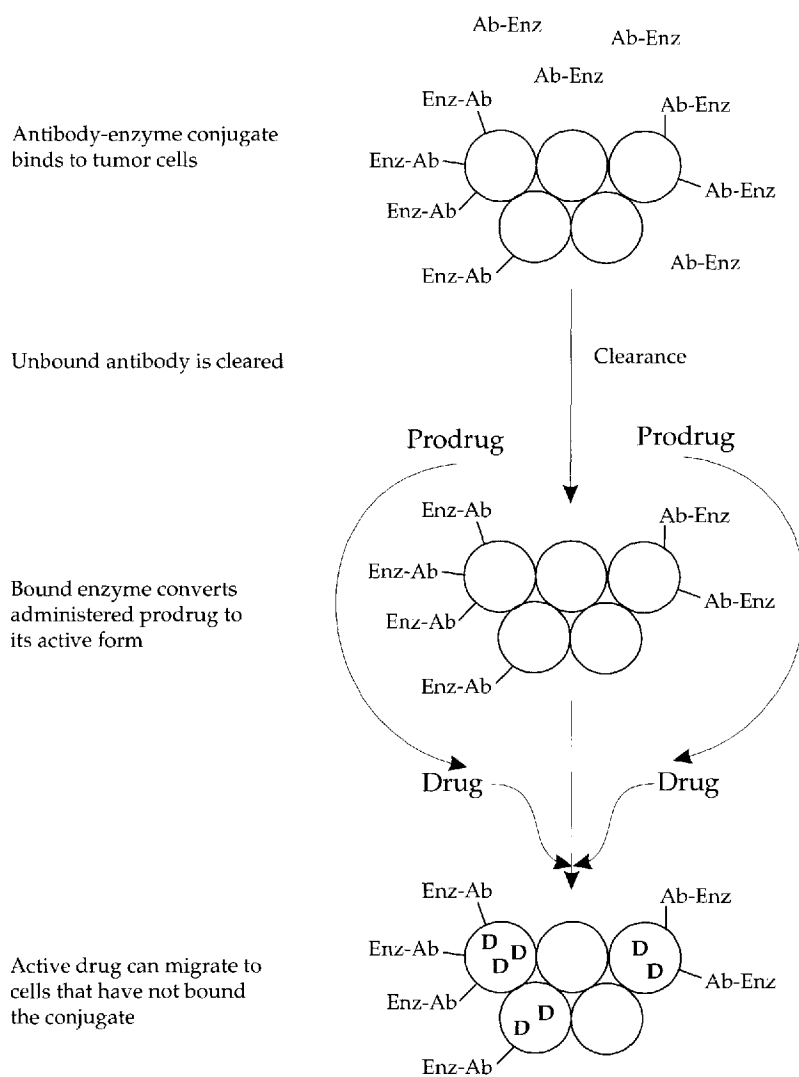Active drug can migrate to cells that have not bound the conjugate

Figure 5. The generation of a cytotoxic drug by ADEPT. In the first phase the antibody-enzyme conjugate is allowed to bind to the target cell population. After unbound conjugate is allowed to clear a prodrug is administered which is converted to an active drug (D) by the bound enzyme. Importantly the active drug can migrate and have cytotoxic effects on cells that have not bound the conjugate.
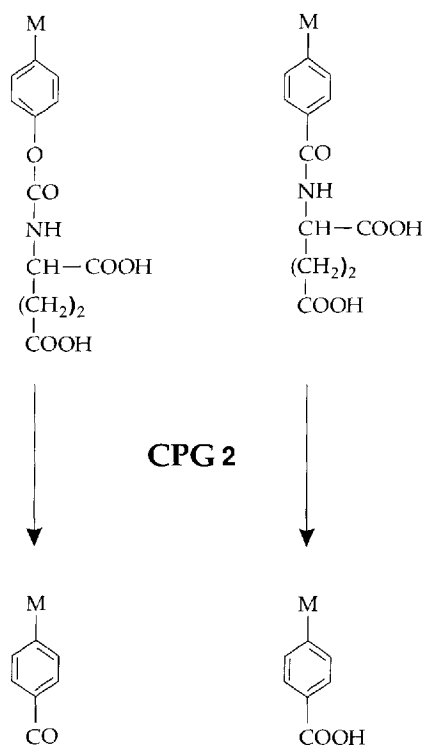
Figure 6. The activation of glutamate prodrugs to active mustards by the enzyme carboxypeptidase G2 (CPG2). M = N(CH₂CH₂Cl)₂.

Gene-Directed Enzyme Prodrug Therapy (GDEPT)

A new gene therapy based approach for targeting cancer cells and making them sensitive to prodrugs has been proposed for human gene therapy trials.[33,34] Prodrug gene therapy, commonly referred to as GDEPT (gene-directed enzyme prodrug therapy) or virus-directed enzyme prodrug therapy (VDEPT) is based on the premise that a large therapeutic benefit can be gained by transferring into tumor cells a drug susceptibility gene. The gene encodes an enzyme that can catalyse the activation of a prodrug to its cytotoxic form (Figure 8) and has also been termed a "suicide gene".[35-38] However, in contrast to cytotoxic gene therapy approaches that involve expression of a toxic product (for example, diphtheria toxin[39-42]), the enzyme itself is not toxic. Thus, in GDEPT, cytotoxicity only results after administration of the prodrug. There is also a bystander effect as the active drug can migrate into non-transduced cells.

GDEPT is obviously related to antibody-directed enzyme prodrug therapy (ADEPT) in that both use an non-endogenous enzyme to activate a prodrug. However, there are fundamental differences between the two techniques and this means that the enzyme/prodrug combinations used for ADEPT may not be suitable for GDEPT. In GDEPT the prodrug activating enzyme is expressed inside the cell whilst in ADEPT it is extracellular. Thus, for

GDEPT the prodrug must be able to enter the cell. Therefore, enzyme prodrug systems developed for ADEPT that rely on a charged prodrug (such as a glucuronide) being excluded from the cell whilst the active drug is not are not directly applicable to GDEPT. However, it should be noted that a GDEPT system that allows for extracellular expression of the activating enzyme has now been described. As with ADEPT, a number of different enzyme/prodrug combinations have been proposed for GDEPT. Again, as in ADEPT, an enzyme is required for which there is no endogenous activity. Bacterial or viral enzymes are particularly suited to GDEPT. Firstly, many of these enzymes have no corresponding activity in humans. Secondly, as expression is intracellular, there should be no problems with an immune response against a 'foreign' protein. Thirdly, bacterial enzymes tend to have few requirements for post translational modification and more complex proteins may not fold correctly in different species. Because activation takes place intracellularly, the prodrug should be freely diffusible and thus a neutral species with an appropriate partition coefficient. As not all of the tumor cells will be transduced by the vector a bystander effect is required. Thus, the active drug should be able to diffuse away from the site of activation. However, its half-life needs to be such that it can not migrate and affect normal tissue. The active drug should also not be phase specific of proliferation dependant since many tumors have a low mitotic index[43] and all malignant cells need to be killed and not just those that are proliferating.
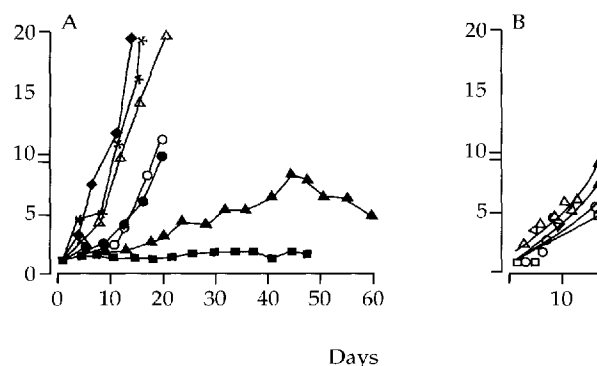

Figure 7. The effect of ADEPT therapy on the growth of a xenograft in vivo. Groups of mice bearing the CC3 choriocarcinoma xenograft were treated (A) intravenously with a W14 F(ab')2: Carboxypeptidase G2 conjugate followed 56, 72 and 80h later with either 5mg/kg (▲) or 10 mg/kg (■) of p-N-bis(2-chloroethyl)aminobenzoyl glutamic acid prodrug. This can be compared with conventional therapy with either 5mg/kg methotrexate (▽), 50mg/kg hydroxyurea (◆), 7.5 μg/ml actinomycin D (●), 20mg/kg cyclophosphamide (*) or 20mg/kg ara-C (○). (B) the mice were treated with either saline (△), conjugate alone (○), prodrug alone ( ) or active drug alone (▽) (both at their maximum tolerated doses).

KNOX and CONNORS

Vector introduces the gene coding for an enzyme into a cell

Transcription
& Translation
E E
E E E E

Enzyme can activate
a produg

PRODRUG   PRODRUG

E   E
DRUG DRUG

Active drug can migrate into
non-infected cells

D   D   D   D   D
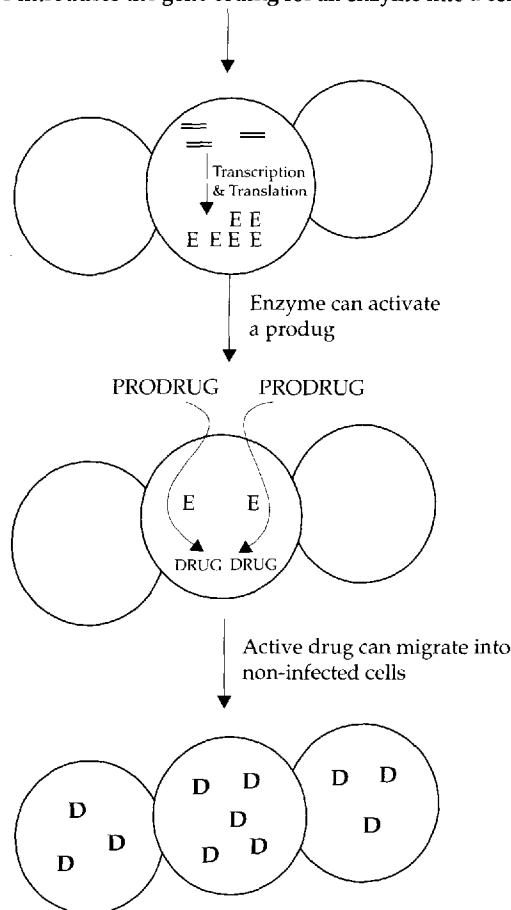D   D   D
D   D   D   D

Figure 8. The generation of a cytotoxic drug by GDEPT. In the first phase the cell is transduced with gene coding for a prodrug activating enzyme. This enzyme (E) is expressed and a prodrug is administered which is converted to an active drug (D) by the bound enzyme. Importantly the active drug can migrate and have cytotoxic effects on cells that have not been transduced.

Various enzyme prodrug systems have been described for GDEPT *(Table 1)* and these are discussed below. However, success for this technique requires not only a choice of an enzyme/prodrug system but also a delivery system by which the gene encoding for the enzyme can be delivered be delivered efficiently and accurately to a human tumor. This aspect is common to all types of gene therapy and has been very well reviewed.[44] At present, targeting is achieved by altering the surface components of viruses and liposomes so as to achieve a level of target cell recognition. Transcriptional elements can be incorporated so that the incorporated gene is expressed only in the target cells. Retroviral based vectors can only infect replicating target cells but integration occurs leading to stable expression of the transduced gene. Adenoviral vectors are more efficient at transduction and do not require

cell division but do not integrate so it is very difficult to regulate gene expression. However, for the long term success of GDEPT (and any gene therapy procedure) there needs to be advances in these systems particularly to improve their targeting. It has been proposed that future gene therapy vectors will be not based on any single virus but will be synthetic vehicles, custom designed to incorporate specific targeting features relevant to the target disease and tissue.[44]

In fact, GDEPT is a recognition of the limitations of the present delivery systems. In comparison to cytotoxic gene therapy, less accuracy is required from the vector because some tumor specificity can be conferred by the pharmacology of the prodrug and its route of administration. Efficiency is improved because is a bystander effect that can kill non-transduced tumor cells. Long term stability of the gene is not required as transduced cells will be killed after prodrug administration. Further, in contrast to all other types of gene therapy, GDEPT is controllable. The expressed enzyme is non-toxic and cytotoxicity can only occur after administration of the prodrug. Therefore, unexpected toxicity can be countered by lowering the dose of the prodrug.

The enzymes and prodrugs used for GDEPT are:

1. Varicella-zoster virus thymidine kinase (VZV-tk)

Varicella-zoster virus produces a thymidine kinase (VZV-tk) with a substrate specificity that is distinct from normal mammalian cellular kinases. VZV-tk can selectively monophosphorylate 6-methoxypurine arabinoside (ara-M). The monophosphate is further metabolised by cellular enzymes (nucleoside diphosphate kinase, AMP kinase, AMP deaminase, adenylosuccinate sythetase lyase) to the cytotoxic adenine arabinonucleoside triphosphate (ara-ATP), a compound that inhibits DNA synthesis. Only very low levels of the triphosphate are detected in mammalian cells in the absence of VZV-tk.[45]

Retroviruses were constructed containing the VZV-tk gene, transcriptionally regulated by either the hepatoma-associated α-fetoprotein or liver-associated albumin transcriptional regulatory sequences. Thus, subsequent to infection by the retrovirus, expression of VZV-tk protein was limited to either α-fetoprotein or albumin positive cells. Cells did selectively express the protein and thus were selectively sensitive to araM.[45] Un-transduced cells could withstand >2000 µM araM. In transduced cells, sensitivity to the prodrug was proportional to VZV-tk activity and was less than 0.5 µM in a high expressing clone.[45] The above system was proposed as a model for the treatment of hepatocellular carcinoma.[45] However, no further work has been published on this system and it appears to have been superseded by the conceptually similar HSV-tk systems described below.

2. Herpes simplex virus thymidine kinase (HSV-tk)

Herpes simplex virus type-1 also expresses a thymidine kinase (HSV-tk) with a substrate specificity different from cellular kinases. HSV-tk converts the purine nucleoside analogues ganciclovir (9-[2-hydroxy-1(hydroxymethyl) ethoxy]methyl guanine), acyclovir (9-[2-hydroxyethoxy] guanine) and FIAU (1[2-deoxy-2-fluoro—D-arabinofura-nosyl]-5-iodouracil) into monophosphorylated compounds. Mammalian kinases can covert these into nucleotide triphosphates that can inhibit DNA replication and act as a chain terminator for DNA synthesis. However, the prodrugs are only poor substrates for mammalian nucleotide monophosphate kinase and these compounds have been used clinically for the treatment of herpes.

HSV-tk/gancilovir is the most described GDEPT system and delivery of the gene coding for HSV-tk to animal tumors in vivo has been achieved using retroviruses, adenoviruses and naked DNA and retroviral vectors are being used to deliver the gene to intracranial and leptomeningeal tumors in rats and the system is proposed for the first clinical trials of GDEPT[33,34] Transfer of HSV-tk in tumor cells confers sensitivity to ganciclovir and its analogues both in vitro and in vivo (Figure 9). In vitro, cells expressing HSV-tk are generally inhibited by 1-50 µM ganciclovir and these levels can be achieved by patients treated with the drug.[46-55] In the absence of prodrug, expression of HSV-tk is not detrimental and expressing cells grow normally in vivo and in vitro.

A clinically relevant model for GDEPT has been developed for brain tumors. These tumors are a good model because apart from the tumor the cellular elements of the brain are non-dividing. Since dividing cells are more easily transduced, the tumor cells would be targeted more directly. Established gliomas in rats could be cured if they had been transfected with the HSV-tk gene and subsequently treated with gancilovir. Rats with brain tumors established by intra-cerebral injection of wild-type or HSV-tk modified glioma cells or by a mixture of wild-type and HSV-tk-modified cells were studied with and without ganciclovir treatments. All rats with HSV-tk expressing tumors treated with ganciclovir survived 90 days while all untreated rats died within 25 days. Further, tumors composed of combinations of 9L and 9L-TK cells could be eliminated by ganciclovir treatments even when only one-half of the tumor cells carried the HSV-tk gene.[49] Rats with a cerebral glioma were also given an intratumoral injection of murine fibroblasts that were producing a retroviral vector in which the HSV-tk had been inserted. The HSV-tk retroviral vectors that were produced transduced the neighbouring proliferating glioma cells.[56,57] The fibroblasts were necessary to attain effective retrovirus titres but rat glioma cells could be transduced directly in vivo using an adenovirus based vector.[51] Therapeutic results have also been obtained with

tumors derived from other malignancies such as liver,[58] breast,[59] head and neck squamous cell cancer,[60] lung,[52,61] ovary[54,55] and melanoma.[50,62]

A bystander effect has been demonstrated for the HSV-tk system. As the active metabolite of ganciclovir, the triphosphate, is highly charged it should not diffuse out of expressing cells and thus it would not be predicted that the bystander effect is mediated by a diffusible metabolite. In vitro, a mixed population containing only 10% of HSV-tk expressing cells was totally killed by a dose of ganciclovir that is not cytotoxic to non-HSV-tk expressing cells.[63] Metabolic co-operation is involved and a ganciclovir metabolic product, presumably a phosphorylated form, can pass from HSV-tk expressing cells to non-expressing cells and mediate cytotoxicity but only as a consequence of direct contact.[64,65] The uptake of apoptotic vessels by the non-expressing tumor cells has also been demonstrated.[63] In vivo, there is also a cell-mediated immune component to the bystander effect.[66]

3. Cytosine Deaminase

This enzyme is found in many fungi and bacteria and catalyses the deamination of cytosine to uracil. It is not found in mammalian cells. Cytosine Deaminase can also convert the clinically used anti-fungal agent 5-fluorocytosine (5-FC) into the known anti-tumor drug 5-fluorouracil (5-FU). 5-FU is further metabolised to 5-flurouridine 5'-triphosphate, 5-fluro-2'-deoxyuridine 5'-triphosphate and 5-fluro-2'-deoxyuridine 5'-monophosphate. The first two compounds inhibit RNA and DNA synthesis respectively whist the monophosphate is a potent inhibitor of thymidylate synthase that is an important enzyme in DNA biosynthesis.

Mouse 3T3 cells were shown to be sensitive to 5-FC when tranfected with the gene encoding cytosine deaminase.[67] When injected into syngeneic mice, the cytosine deaminase expressing tumors could be eliminated in vivo by systemic treatment with 5-fluorocytosine without significant toxicity to the host, although delaying the prodrug treatment reduced its effectiveness. Interestingly, in animals whose tumors had been eliminated by prodrug treatment, they resisted subsequent re-challenge with unmodified wild type tumor. This post-treatment immunity appeared to be tumor specific.[37] It was postulated that cell death lead to a more effective antigen presentation or that the cytosine deaminase protein itself was immunogenic.[37] Human colorectal tumors were also shown to be sensitive to 5-FC when they expressed cytosine deaminase.[68] The sensitivity of these cells was retained in vivo when grown in nude mice.[68] Importantly, a large bystander effect was observed both in vitro and in vivo.[68] 5-FU was liberated into the surrounding environment when cytosine deaminase-expressing tumor cells are treated with 5FC. This lib-

erated 5FU is able to kill neighbouring, non-cytosine deaminase-expressing tumor cells *in vitro* and *in vivo*.[69] When only 2% of the tumor mass contained cytosine deaminase-expressing cells (98% non-cytosine deaminase-expressing cells), significant regressions in tumors were observed when the host mouse is dosed with non-toxic levels of 5FC.[69]

In a comparison of cytosine deaminase with HSV-tk, transduced into human lung adenocarcinoma cell lines and driven by the CMV promoter, cytosine deaminase was superior in its ability to achieve high levels of specific enzyme activity, to induce growth inhibition and to affect neighbouring cell growth.[70]

4. Cytochrome-P450

Cyclophosphamide is a commonly used anti-tumor agent. It is a prodrug that is activated by microsomal mixed-function oxidases cytochrome-P450 to form phosphoramide mustard via the 4-hydroxy intermediate that exists in equilibrium with the ring opened tautomer, aldophosphamide. Cytochrome P-450 is a complex system involving many isoenzymes, not all of which can activate cyclophosphamide. In humans, the isoenzymes 3A4 and 2B6 are mainly responsible for the activation of cyclophosphamide. These enzymes are only present in liver and not in other tissues or in tumor cells. Thus, there would appear be a rationale for introducing a cytochrome P-450 into a tumor cell. However, the 4-hydroxy/aldophosphamide intermediate is a circulating metabolite and is known to reach tumor cells. Therefore, this systemic activation of the prodrug might mitigate any selective activation by an introduced gene.[71] As predicted, tumor cells can be sensitised to the cytotoxic effects of cyclophosphamide, in culture, by introduction of cytochrome P450 2B1. Stable transfection of rat C6 glioma cells with the P450 2B1 gene rendered the cultured tumor cells sensitive to cyclophosphamide.[72] Further, C6 cells bearing this gene were more sensitive than parental cells to the cytotoxic action of CPA when grown subcutaneously in the flanks of athymic mice.[72] Similar results were seen with rat 9L gliosarcoma cells[73,74] and human MCF-7 cells.[75] *In vitro*, a panel of human tumors was shown to be sensitised to cyclophosphamide and its analogue ifosfamide when infected with the cytochrome P450 gene.[76]

Thus, it has been shown that expression of cytochrome P450 can significantly increase the anti-tumor efficacy of cyclophosphamide without any increase in host toxicity. Therefore, there is a substantial 'proximity effect' with intra-tumoral cyclophosphamide activation despite the high metabolic activation by the liver.[73] This could suggest that the circulating metabolite (the 4-hydroxy/aldophosphamide intermediate) has either limited access to the

tumor vasculature or a low degree of cell permeability. Alternatively it has been suggested that acrolein, which is formed in equimolar amounts with phosphoramide mustard can potentiate the cytotoxicity of the mustard locally.[73] Acrolein is a very reactive compound and reacts rapidly with proteins and thiols. Thus it is rapidly lost from the circulation. However, local generation could result in the depletion of thiols such as glutathione and thereby modulate the cytotoxicity of phosphoramide mustard.

A bystander effect was observed with this system and is mediated by diffusible, cytotoxic, metabolite.[73,75,77]

5. Carboxypeptidase G2

Carboxypeptidase G2 (CPG2) was originally isolated from *Pseudomonas sp* RS-16 and is a dimeric protein (Mr 83 kDa) consisting of two identical, zinc containing, subunits. It catalyses the hydrolysis and cleavage of folates to pteroates and L-glutamic acid but can also cleave other substrates that are prodrugs such as the mustard derivatives of glutamic acid (*Figure 6*). The enzyme has no equivalent mammalian activity. This enzyme and potential prodrugs have been extensively studied for ADEPT in which it has shown excellent results (see above). However, the properties of both the enzyme and its prodrugs would appear to mitigate its use in GDEPT. Firstly, because the enzyme can degrade folates, expression of CPG2 inside a cell may be cytotoxic because it can deplete folate pools.[78] Secondly, because the prodrugs are derivatives of glutamic acid, they are charged and would not be expected to readily cross a cell membrane. The reactivity of the mustard is reduced by the linkage of the benzoic acid moiety through an amide bond to a glutamic acid residue. However, this deactivation was not thought to be great enough to account for the difference in cytotoxicity. This enhanced effect has been ascribed to the fact that the prodrug is excluded from the cell. In ADEPT this is an ideal situation because the activating enzyme is localised to the cell surface. However, in GDEPT the enzyme is intracellular and would not be available to activate the prodrug.

Contrary to the theoretical considerations, the expression of CPG2 in combination with the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic (CMDA) would appear to be an effective GDEPT system.[79] Because CPG2 is a secreted protein and the enzyme is found in the bacterial periplasm[80] the bacterial sequence was engineered to remove the signal peptide from the gene. This gene was introduced into a number of cell lines by transfection under the control under the control of a non-specific promoter from the elongation 1 α gene. Stable clones were obtained which expressed CPG2 and which were sensitive to CMDA and some clones were nearly 100-fold more sensitive than the corresponding control cell lines.[79] There was no reduction in the rate of

growth of the expressing cells and therefore CPG2 was not toxic to these cells. However, normal tissue culture medium is rich in thymidine and can rescue cells from folate depletion.

A large bystander effect was also observed.[79] When CPG2 expressing LS174T cells were mixed with non-expressing cells, a total kills of the whole population occurred when only ~5% where expressing CPG2.

Interestingly, the extracellular expression of CPG2 for GDEPT applications has also been reported.[81] The gene was engineered so it was expressed tethered to the outer surface of mammalian cells. It was found necessary to mutate the gene so as remove some glycosylation sites as glycosylation was found to inhibit the enzyme (proteins in bacteria are not normally glycosylated). Whilst this reduced the efficiency of surface expression, extracellular expression was achieved. Results obtained with the CMDA prodrug, with respect to cytotoxicity and bystander effect were very similar to those obtained with intracellular expression of CPG2.[81] Whilst glycosylation was a problem for the external expression of CPG2, this might not be the case with other enzymes and this method would allow for the use of any of the enzyme/prodrug systems developed for ADEPT to be used in GDEPT. However, external expression of an enzyme may stimulate an immune response against it. Whilst this would probably inhibit prodrug activation it may lead to an anti-tumor response in its own right. This combination of immunotherapy and GDEPT may have advantages but puts extra constraints on the targeting system.

6. Nitroreductase

Like carboxypeptidase G2, nitroreductase (NR) was originally proposed as an enzyme for use in ADEPT.[2] NR was isolated and cloned from $E\ coli$[82] and is a flavoenzyme (containing) FMN, and is a 23kDa monomer.[83,84] NR can activate the prodrug CB1954, (5-(aziridin-1-yl)-2,4-dinitrobenzamide), by reducing either of the nitro groups of CB 1954 and forms a mixture of the 2- and 4-hydroxylamines in equal proportions.[84] 5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, can react chemically with cellular thioesters (such as S-acetylthiocholine) to a highly reactive DNA-crosslinking species [1,85] (see Figure 1). 5-(Aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide is not as cytotoxic as the 4-hydroxylamine and is a less potent DNA crosslinking agent. However, it is still much more cytotoxic than the prodrug, CB 1954. Thus CB 1954, is converted from a weak mono-functional alkylating agent to a potent difunctional agent (which can form DNA interstrand crosslinks). There is a very large difference between the cytotoxicity of the prodrug and the active form (10,000) of CB 1954,[3] which makes this system attractive for GDEPT. In human cells, CB 1954 can be

reduced by the enzyme DT diaphorase but reduction is very slow (Km=1.4mM, k_{cat}=0.009s^{-1})[10] compared to NR (Km=1.4mM, k_{cat}=6.0s^{-1})[86] and human cells (even those expressing high levels of human DT-diaphorase) are resistant to CB 1954.[10] The application of nitroreductase in ADEPT was handicapped by its requirement for a cofactor.[2,87] However, in GDEPT this is not a problem because the enzyme is expressed intracellularly and thus can use the endogenous cofactors NADH or NADPH (NR can use either[86]). In fact, the cofactor requirement may be an advantage because any enzyme that escapes into the circulation (for example, from dying cells) will be incapable of activating circulating prodrug because of the lack of a cofactor. This is because NAD(P)H is very rapidly metabolised by serum components.[88]

A recombinant retrovirus encoding NR was used to infect mammalian cells. NIH3T3 cells expressing NR were killed by CB1954. The bulk infected, unselected, cell population were about a 100-fold more sensitive to CB 1954 than the parental cells.[89] A selected clone was even more sensitive and, using a cell count assay, was over a 1000-fold more sensitive to CB 1954 than parental NIH3T3 cells (Figure 9).[89] Similar results were seen in human melanoma, ovarian carcinoma and mesothelioma cells.[89] The rapid action of CB 1954 and the resulting need for a shorter exposure time may facilitate the use of this prodrug clinically.[90] A significant bystander effect
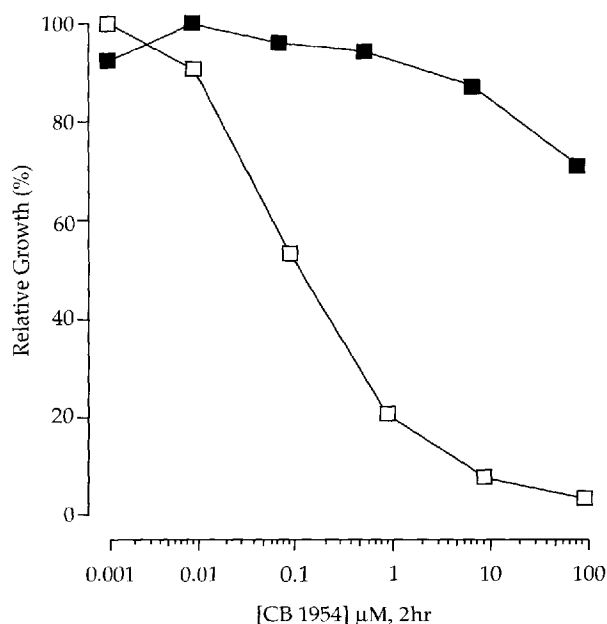
Figure 9. The cytotoxicity of CB 1954 against a nitroreductase (NR) expressing cell line. NIH3T3 cells were infected with a recombinant retrovirus containing NR and a cell clone (NIH3T3-NR) derived by limiting dilution. Parental NIH3T3 cells (■) or NIH3T3-NR cells (□) were treated with CB 1954 for 24h prior to assay.

was observed and admixed, unmodified NIH3T3 cells, could also be killed by a normally non-toxic dose of pro-drug.[89] The bystander effect is mitigated by diffusible metabolites and both the 2- and 4-hydroxylamino derivatives of CB 1954 are release into the medium of CB 1954 treated nitroreductase-expressing NIH3T3 cells.[91] Importantly, and in contrast to the Herpes simplex virus thymidine kinase/ganciclovir enzyme/prodrug system, NR/CB1954 cell killing was cytotoxic towards non-cycling cells.[89]

As with ADEPT, other potential prodrugs have been proposed for use with the nitroreductase. Chinese hamster V79 cells transfected with a nitroreductase expression vector were 770-fold more sensitive to CB1954 than control non-expressing cells. Other prodrugs such as nitrofurazone (97-fold) and the nitroimidazole compounds, misonidazole (21-fold) and metronidazole (50-fold) also exhibited increased cytotoxicity against the nitroreductase-expressing cells and were found by HPLC to act as substrates for the purified NR enzyme.[92] However, this correlation was not absolute. In particular the quinone EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2-(H-indole-4, 7-indione)-propenol) showed only a very small differential (<3-fold).[92] This is probably because this compound can undergo activation by endogenous enzymes such as NADPH: cytochrome P450 reductase.[92] When misonidazole or metronidazole were activated by NR extracellularly, there was little increase in cytotoxicity.[83] This would suggest that the active species has a very short half-life and probably would not exhibit a large bystander effect. A series of 2,4-dinitrobenzamide mustard analogues of CB 1954 have also been evaluated as potential prodrugs for GDEPT in a V79 cell line transfected with the nitroreductase.[93] Other potential prodrugs that could be used in GDEPT are those are those activated by a self-immolative mechanism (*Figure 10*) which can potentially form active drugs such as mustards, actinomycins, mitomycin C,[94] enediynes,[95] seco-CI alkylating agents[96] or tallimustine.[97] The prodrugs are 4-nitrobenzyloxycarbonyl derivatives of these drugs, which upon enzymatic reduction, generated the drug through self-immolation of the 4-(hydroxylamino)benzyloxycarbonyl group. Prodrugs of this type have been synthesised and shown to be activated by NR[94-97] but have not yet been tested against a NR expressing cell line.

7. Other enzymes

Mention should also be made of two other enzymes have been considered in a GDEPT context; thymidine phosphorylase[98] and deoxycytidine kinase.[99] However, they differ from the above examples because they are normal human enzymes. Resistance to the prodrugs they activate may be due to low expression of these enzymes in the
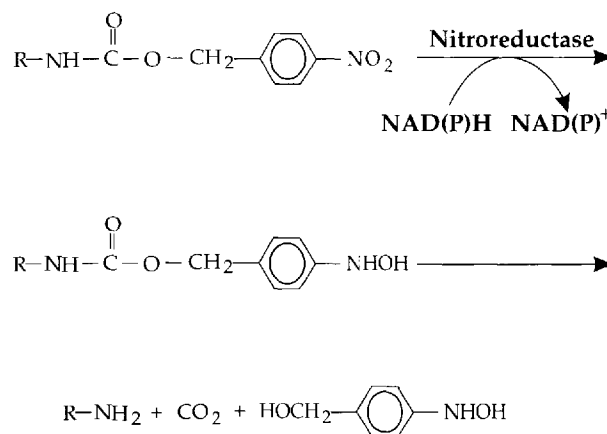

Figure 10. The generation of an active drug (R-NH2) by self-immolation of a prodrug after nitroreduction. As well as carmbamates, carbonates can also undergo reduction and self-immolation to form an active drug (R-OH).

tumor and thus a therapeutic advantage could be gained by their selective expression.

Human thymidine phosphorylase (dThdPase) is identical to platelet-derived endothelial cell growth factor (PD-ECGF) and catalyses the reversible phosphorolytic cleavage of thymidine, deoxyuridine and their analogues to the base and deoxyribose 1-phosphate. It can also cleave the cleave the glycosidic bond of the prodrug 5'-deoxy-5-fluorouridine (5'-DFUR) to form 5-fluorouracil (5-FU). In some tumors resistance, to 5'-DFUR therapy could be due to low dThdPase activity, and thus GDEPT could raise the dThdPase levels and thus enhance sensitivity to the prodrug. Human MCF-7 breast cancer cell lines had up to a 165-fold increased sensitivity to the prodrug in vitro when transfected with the dThdPase cDNA. Sensitivity to 5-FU and 5-fluoro-2'-deoxyuridine were unchanged.[98]

Cytosine arabinoside (ara-C) is a cytidine analogue that incorporates into replicating DNA and induces lethal DNA strand breaks. Although ara-C is a potent anti-tumor agent for haematological malignancies, and is the most effective agent in the treatment of acute myeloid leukaemia, it has only minimal activity against most solid tumors. The rate-limiting step in intracellular ara-C activation is phosphorylation of the prodrug by deoxycytidine kinase (dCK) which is in competition with the detoxification of the prodrug by deamination by cytidine deaminase. Thus it was considered that solid tumors could be made more sensitive to the prodrug if the balance was shifted from the detoxification pathway and to the activation pathway by introducing dCK.[99] Both retroviral and adenoviral vector-mediated transduction of the dCK cDNA resulted in the sensitisation (~10-fold after a 96h prodrug exposure) of the 9L rat glioma cell line to the cytotoxic effects of ara-C in vitro.[99]

All of the above examples demonstrate that selective killing of tumor cells can be achieved by GDEPT. Perhaps unfortunately, many of the active drugs formed are antimetabolites. These are only cytotoxic to dividing cells and tend to require long exposure times for optimum effect. Alkylating agents do not suffer from these disadvantages and are also less prone to induce drug resistance.[100] The generation of an alkylating agent probably offers the most potent means of killing targeted cell types. However, given the different modes of action of, for example, nitroreductase/CB 1954 and HSV-tk/ganciclovir, a combination of these approaches offers a way of obtaining potentially synergistic effects. Co-operative killing was observed when cells expressing both NR and HSV-tk were treated with a combination of CB1954 and ganciclovir.[89]

A bystander effect is also required for the active agent. This is because the present gene therapy vectors can not transduce all the target tumor cells. In some models, less than 1% of genetically modified cells can produce significant cytotoxicity in the bulk population.[93] In most cases, the bystander effect is mitigated by diffusible cytotoxic metabolites formed by prodrug activation. In the case of the purine nucleotides, a direct cell-cell contact is involved and gap junctions are involved in the transfer of the cytotoxic metabolites. However, other factors may be involved and it has been postulated that lytic enzymes, triggered by programmed cell death, can also be transferred.[63] In vivo, the immune system is also involved and there may even be a priming effect.[63,66]

Conclusions

Despite many excellent examples of prodrug activating systems, the direct use of prodrugs in the clinic has been disappointing mainly because the correct profile of activating enzymes has not been found. However, comparison of biochemical, molecular biological, and chemosensitivity data obtained from screening a large number of cell lines (e.g., the NCI tumor cell line panel) may facilitate investigation of factors influencing drug anti-tumor activity. The knowledge gained may be of value in the development of new anti-cancer agents or in the selection of patients to receive specific therapies.[101]

Two new therapies have been proposed to overcome limitations of prodrug therapy. In ADEPT, a cytotoxic agent is generated selectively at the site of a tumor by an antibody targeted enzyme. The antibody delivery combined with the amplification provided by the enzymatic activation of prodrugs enables adequate selection to be made between tumor and normal tissue. In GDEPT, specific promoters can be used to allow selective expression of an activating in a tumor after it has been infected using, for example, a retroviral vector. Both ADEPT and GDEPT have been proven to be very effective in animal models

and there is a rich diversity of potential enzyme and prodrug combinations for use in these. Both ADEPT and GDEPT offer new research ideas for the chemist, enzymologist, molecular biologist and protein engineer as well as the cancer chemotherapist. However, systemic administration of the present generation of gene therapy vectors is not possible and GDEPT, unlike ADEPT, is thus limited to isolable tumor deposits, such as intracerebral tumors, surrounded by largely non-dividing normal tissue. In such cases, GDEPT is feasible using retroviral based vectors. Improved vectors have been proposed and are certainly under development. On the other hand, it should be remembered that GDEPT is an answer to some of the limitations of the present gene therapy vectors and a perfect, tumor specific, gene delivery system would make GDEPT obsolete. In contrast, ADEPT offer opportunities for the therapy of systemic cancer and may be a major advance for the treatment of solid tumors. ADEPT has shown encouraging results in a preliminary clinical trial. The results of more extensive trials are awaited with interest and ADEPT systems may soon form an important element of the armament of the clinical oncologist.

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