J. Pharm. Pharmacol. 1998, 50: 387–394
Received September 24, 1997

The Design of Selectively-activated Anti-cancer Prodrugs for use in Antibody-directed and Gene-directed Enzyme-Prodrug Therapies*

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Abstract

Systemic anti-proliferative agents (cytotoxins) have been the most successful single design concept for anti-cancer drugs. However, they have inherent limitations (they target dividing cells rather than cancer cells) which limit their clinical efficacy, especially toward the more slowly-growing solid tumours. New concepts are required to improve the selectivity of their killing of tumour cells.

One possibility is the use of prodrugs which can be activated selectively in tumour tissue. Several potential mechanisms for this are being explored, including tumour hypoxia, low extracellular pH, therapeutic radiation and tumour-specific endogenous or exogenous enzymes. In the last approach the exogenous enzyme can be delivered by attachment to monoclonal antibodies (ADEPT) or as DNA constructs containing the corresponding gene (GDEPT). A limitation of both approaches is that only a small proportion of the tumour cells become activation-competent, but this can be substantially overcome by the design of appropriate prodrugs capable of killing activation-incompetent cells via a bystander effect.

We have proposed a modular approach to prodrug design in which a trigger unit determines tumour selectivity and an effector unit achieves the desired level of killing of cells when the trigger is activated. For ADEPT and GDEPT prodrugs the primary requirement of the trigger is efficient and selective activation by the appropriate enzyme; the released effector must be a potent, diffusible cytotoxin which fully exploits the small proportion of cells capable of activating the prodrug. A wide variety of chemistries has been used, but many of the existing effectors do not have all of these properties. We report work on two types of cytotoxin derived from very potent anti-tumour antibiotics (enediynes and amino-*seco*-cyclopropylindolines) as effectors in prodrugs for ADEPT and GDEPT applications.

Most current anti-cancer drugs are systemic anti-proliferative agents (cytotoxins) which are distributed widely in the body and preferentially kill dividing cells, primarily by attacking their DNA at some level (synthesis, replication or processing). Although this has been the most successful single design concept for anti-cancer drugs, the efficacy of these drugs is generally limited by the damage they also cause to proliferating normal cells, such as those in the bone marrow and gut epithelia. Further improvements in this general class of systemic anti-proliferative agent can be expected, especially in the capacities of individual compounds to overcome the common mechanisms of cellular resistance. However, to achieve substantial increases in the clinical efficacy of such drugs, especially against the more slowly-growing and common solid tumours, the selectivity of the killing of tumour cells must be improved by development of new concepts. One such concept, aspects of which are discussed here, is the development of prodrug forms which can be activated selectively in tumour tissue.

*Presented at the British Pharmaceutical Conference, Scarborough, September 15–18, 1997.

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Mechanisms for the Tumour-selective Activation of Prodrugs

Prodrugs are compounds that are transformed after administration, either by metabolism or by spontaneous chemical breakdown, to form the pharmacologically active species. Prodrugs are currently used in cancer chemotherapy (perhaps the best-known example is cyclophosphamide), but these prodrugs usually undergo non-specific activation, and serve primarily to modify drug uptake or pharmacokinetics by controlling properties such as lipophilicity. Recently, several more tumour-selective approaches to prodrug activation have been identified; these have generated renewed interest in the prodrug concept for anti-cancer therapeutics.

Tumour hypoxia

Inefficient blood vessel networks in solid tumours, resulting from imperfect neovascularization, and high and variable interstitial pressures result in limited and chaotic blood flow. This in turn leads to the presence of a significant proportion of chronically and acutely hypoxic cells with oxygen tensions below ca 0·1% (Coleman 1988). Such hypoxia seems to be a common and unique property of cells in solid tumours and there is extensive interest in prodrugs designed to be activated under hypoxic conditions via oxygen-inhibited bioreduction (Wilson 1992; Siim et al 1997).

Differences in extracellular pH

Lower extracellular pH in solid tumours is another consistent difference between tumour and normal tissues (Tannock & Rotin 1989), arising also from poor blood flow and consequent hypoxia. Inefficient clearance of metabolic acids from chronically hypoxic cells can reduce the mean extracellular pH in tumours to below ca 6·3, up to one pH unit lower than the intracellular pH, which is actively regulated, and cell-excluded prodrugs that can be selectively activated at lower pH have been described (Tietze et al 1989).

Therapeutic radiation

In principle, the reducing species produced from the radiolysis of water by ionizing radiation can be used to activate prodrugs in an oxygen-inhibitable manner, restricting activation to the hypoxic cells in the radiation field (Wilson et al 1996). Radiation therapy is widely used in cancer treatment, and modern equipment can deliver a dose of radiation very selectively to solid tumours. A possible drawback of this approach is the small amount of reducing equivalents delivered by a therapeutic dose of radiation, requiring the release of very potent active species (Denny et al 1996; Wilson et al 1996).

Tumour-specific endogenous enzymes

There have been many attempts to develop drugs targeted to 'tumour-specific' enzymes, but in general these efforts have not been very successful. Although prodrugs activated quite specifically by a particular enzyme have been developed, the tumour-specificity of these enzymes is less clear. An early example was the 5-aziridinyl-2,4-dinitrobenzamide CB 1954 (**1**; structures of the compounds discussed are given in Figure 1), which

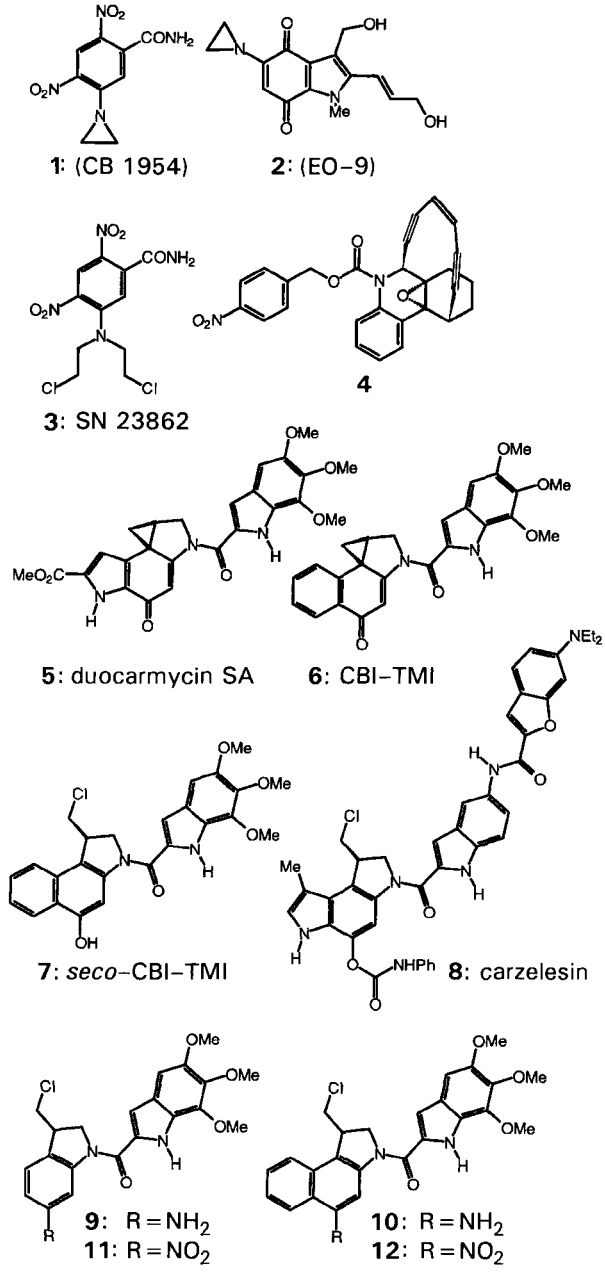

Figure 1. The structures of the compounds discussed.

effected complete cures of the Walker 256 rat carcinoma (Cobb et al 1969). This tumour was found to express a high level of the enzyme DT diaphorase, which activated the prodrug by reduction of the 4-nitro group (Knox et al 1991). In man, however, **1** was a much poorer substrate for DT diaphorase than in the rat (K_{cat} 0·64 min^{-1} compared with 4·1 min^{-1}; Boland et al 1991), and so CB 1954 was not clinically useful. The aziridoquinone EO-9 (**2**) is also specifically activated by DT diaphorase, and its cytotoxicity to a range of tumour lines correlates well with the cellular levels of DT diaphorase (Fitzsimmons et al 1996); again, however, clinical results with EO-9 have so far been disappointing (Dirix et al 1996; Pavlidis et al 1996).

Exogenous enzymes: ADEPT and GDEPT strategies

The potential advantages of using a specific exogenous enzyme to activate a prodrug selectively have resulted in the development of two broad methodologies. In ADEPT (antibody-directed enzyme-prodrug therapy), the exogenous enzyme is targeted selectively to tumour cells by linking it to a monoclonal antibody directed against a tumour-specific epitope (Bagshawe et al. 1994). In GDEPT (gene-directed enzyme-prodrug therapy) the exogenous enzyme is generated selectively in the tumour cells after delivery of a DNA construct containing the corresponding gene. Tumour selectivity in this approach is a combination of the selectivity of the delivery of the DNA construct (e.g. physically by direct injection of liposomes, or to dividing cells via a retroviral vector) and the selectivity of expression of the gene (controlled by the presence of appropriate promoter or other control sequences, or both, in the construct) (Zhang & Russell 1996).

Although it might be possible to achieve high selectivity of delivery of the exogenous activating enzyme to tumours by these methods, it has not been possible to ensure their delivery to all the tumour cells. In ADEPT many tumour cells express low levels of the target antigen, or none at all, and many of those that do might not be accessible to large antibody-enzyme conjugates with limited diffusional properties. In GDEPT also despite exploration of a variety of methods of gene delivery and expression most protocols result in the generation of exogenous enzyme in only a small proportion of the target cells. However, this drawback is substantially overcome by the catalytic capability of the activating enzyme, and by proper design of the prodrug, as discussed below.

A Concept for the Design of Anti-cancer Prodrugs

Irrespective of the mechanism of their selective activation, prodrugs of systemic anti-proliferative agents (cytotoxins) can be considered as comprising three distinct (although possibly not separate) domains, with trigger and effector units joined by a linker mechanism (Denny 1996; Denny et al 1996; Siim et al 1997) (Figure 2). The trigger unit determines the selectivity of the prodrug by undergoing activation by one of the above tumour-specific mechanisms. The effector unit is masked in the prodrug form, but achieves a desired level of cell killing when the trigger is activated. These two units are linked in such a way that activation of the trigger rapidly generates the cytotoxic effector. The linkage might be a separate and distinct part of the prodrug, or just a mechanism by which activation of the trigger results in changes in the effector group. This concept emphasizes the distinctness of the separate units and the different roles they play. It enables independent consideration of the criteria that each unit must meet and the types of chemical structure that are most suitable.

The trigger unit

This is the primary determinant of the tumour selectivity of the prodrug. Several activating enzymes have been used to activate prodrugs in ADEPT and GDEPT protocols, including carboxypeptidase G2 from *Pseudomonas* species (Springer et al 1995), β-lactamase from *Enterobacter* species (Meyer et al 1992, 1993), β-glucuronidase and β-galactosidase from *E. coli* (Haisma et al 1992; Abraham et al 1994), thymidine kinase from *Herpes simplex* virus (Ishii-Morita et al 1997), cytosine deaminase from various sources (Richards et al 1995; Li et al 1997) and nitroreductase from *E. coli* (Boland et al 1991; Anlezark et al 1992). A common requirement is that the enzymes can activate the chosen prodrug efficiently and selectively.

The effector unit

As noted above, in both ADEPT and GDEPT strategies only a small proportion of tumour cells is likely to be rendered activation-competent, and the amount of effector generated from the prodrug by the enzyme is limited, even though the process is

Figure 2. Modular design of prodrugs.

catalytic. The released effector must therefore have adequate cytotoxicity and be able to kill cells in a variety of pH environments and proliferative states (many cells in solid tumours cycle only very slowly) (Denny & Wilson 1993). Because of this, cytotoxins such as anti-metabolites, topoisomerase inhibitors and mitotic poisons, which are very specific for cycling cells, might be less desirable as effectors than less specific agents such as DNA alkylators and DNA-breaking agents. The released effector must also be able to travel a limited distance from the site of activation to kill surrounding cells (but not so far that it reaches to the systemic circulation). This property, termed the 'bystander effect', requires both a suitable half-life (suggested to be from many seconds to a few minutes; Denny & Wilson 1993) and diffusional ability (generally precluding effectors that bind tightly to macromolecules such as DNA). Effectors with an adequate bystander effect ensure that the small proportion of cells capable of activating the prodrug can be fully exploited, making them foci for the catalytic production of cytotoxic species.

Examples of Prodrugs for ADEPT and GDEPT

The selected examples described below are used to illustrate briefly the above principles of ADEPT and GDEPT approaches, and in particular the design of prodrugs.

Ganciclovir-thymidine kinase

The non-toxic antifungal drug ganciclovir in conjunction with the thymidine kinase enzyme from the *Herpes simplex* virus has been widely used as a prodrug in GDEPT protocols (Figure 3). The enzyme converts the drug selectively to the monophosphate which, although a cell-impermeant species, becomes distributed among surrounding cells by way of gap junctions (Ishii-Morita et al 1997). The monophosphate (but not ganciclovir) can be converted by cellular enzymes to the toxic triphosphate species. This system was one of the

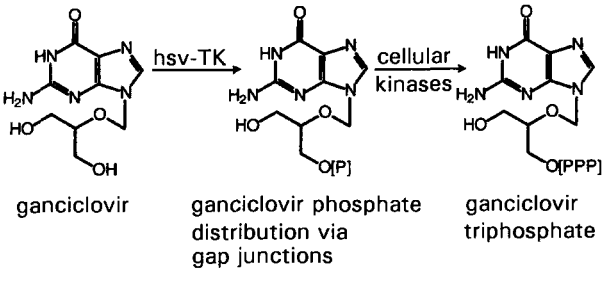

Figure 3. The use of Herpes thymidine kinase with ganciclovir in GDEPT protocols.

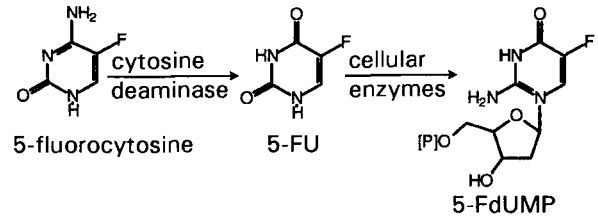

Figure 4. The use of yeast cytosine deaminase with 5-fluorocytosine in GDEPT protocols.

earliest developed, and has undergone several Phase I clinical trials (a review has been written by Culver (1996)), but a detailed evaluation of its clinical effectiveness is not yet available. Possible limitations of this approach are the relatively low cytotoxicity of the effector, and the absence of well-developed gap junctions from many tumours (Holder et al 1993).

5-Fluorocytosine-cytosine deaminase

This example makes use of the capacity of yeast cytosine deaminase to convert the non-toxic 5-fluorocytosine selectively to 5-fluorouracil (Figure 4). This effector, which can diffuse freely, is converted by cellular enzymes to the toxic 5-FdUMP (an inhibitor of thymidylate synthetase). 5-Fluorouracil is one of the most effective drugs for treatment of colon cancer, and studies have been reported on the use of this GDEPT protocol with cytosine deaminase under the control of promotor/regulatory elements for carcinoembryonic antigen (Richards et al 1995). Clinical trials have not yet been reported. Possible drawbacks include the relatively low potency and the cell-cycle selectivity of 5-fluorouracil.

Desacetylvinblastine hydrazide-β-lactamase

The selective capacity of β-lactamase enzymes to hydrolyse the four-membered β-lactam ring of penicillins and cephalosporins, resulting in spontaneous fragmentation of a carbamate side chain and release of amine-based effectors, has been used in a variety of prodrugs. In an ADEPT approach (Figure 5) a cephalosporin prodrug of the vinca

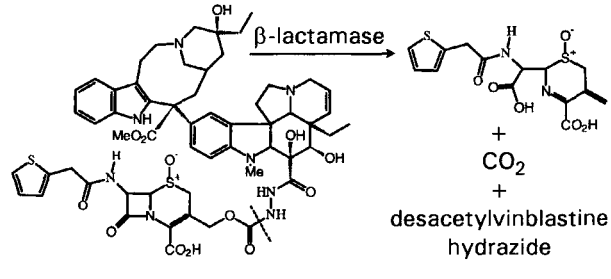

Figure 5. The use of bacterial β-lactamase and the hydrazide of desacetylvinblastine in ADEPT protocols (cleavage at).

alkaloid and mitotic inhibitor desacetylvinblastine hydrazide was shown to be an excellent substrate for β-lactamase-antibody conjugates (Meyer et al 1992). Although the difference between the potencies of prodrug and effector was only 5-fold, the activity of the parent drug towards antigen-positive cells was fully restored by pre-incubation with the immunoconjugate. Although the effector is a cell-cycle specific mitotic poison, and unlikely to be active against the full complement of cells in a solid tumour, this prodrug-immunoconjugate protocol resulted in long-term tumour regression in colon tumour xenograft studies in nude mice (Meyer et al 1993).

Epirubicin glucuronide-β-glucuronidase

To minimize the possibility of immune responses developing in man in response to repeated treatment with foreign proteins in ADEPT, the use has been suggested of enzymes such as β-glucuronidase, which exist in low amounts only in serum (Wang et al 1992). Several approaches have been studied (a review has been written by Hay & Denny (1996)), including the *O*-glucuronide of the topoisomerase II inhibitor epirubicin, an established clinical drug (Figure 6). Although this hydrophilic prodrug was 100- to 1000-fold less toxic than epirubicin in cell culture studies, largely because of cell exclusion, co-treatment of antigen-positive cells with the prodrug and an antibody-*E. coli* glucuronidase conjugate was as cytotoxic as treatment with epirubicin alone (Haisma et al 1992). However, the utility of such tightly DNA-binding, cell-cycle-specific (topo II inhibitor) effectors in ADEPT and GDEPT approaches is not yet known.

Iodomustard glutamate-carboxypeptidase

Aniline mustards have been used extensively as the effectors in ADEPT and GDEPT prodrugs. One of the most widely studied systems has been the use of the bacterial carboxypeptidase G2 enzyme to release 4-substituted aniline mustards from glutamate-type prodrugs (Springer et al 1995). These prodrugs are effectively excluded from cells by the diacid side-chain. Cleavage of this by the enzyme simultaneously enables cellular penetration by the released effector and activates it by electron release

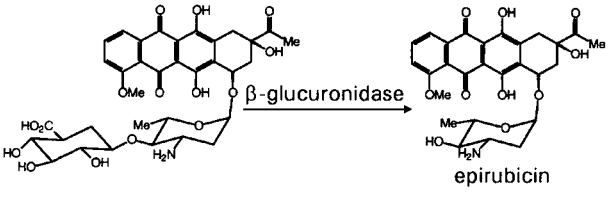

Figure 6. The use of bacterial β-glucuronidase with epirubicin glucuronidate in ADEPT protocols.

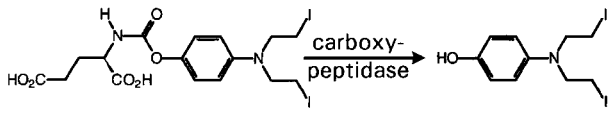

Figure 7. The use of *Pseudomonas* carboxypeptidase G2 and 4-hydroxyaniline iodomustard carbamates in ADEPT protocols.

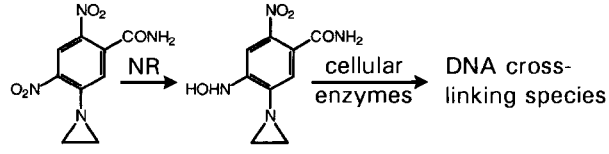

Figure 8. The use of *E. coli* nitroreductase and 5-aziridinyl-2,4-dinitrobenzamide in GDEPT protocols. NR = nitroreductase.

through the aromatic ring to the mustard nitrogen. A limited clinical trial of a carboxylate analogue using an ADEPT protocol resulted in some response (Bagshawe et al 1994). A later carbamate prodrug, releasing a more cytotoxic phenol iodomustard (Figure 7), resulted in growth delays of 14–28 days for LoVo colon carcinoma xenografts in nude mice, in conjunction with a carboxypeptidase G2-carcinoembryonic antigen-antibody conjugate (Blakey et al 1995); clinical trials are planned.

Dinitrobenzamide-nitroreductase (Figure 8)

The aerobic nitroreductase isolated from a strain of *E. coli* has been shown to be more efficient than rat DT diaphorase in the activation of the aziridine **1** (K_{cat} 360 min^{-1}; Boland et al 1991; Anlezark et al 1992), whereas activation of the related mustard **3** (SN 23862) is even more facile (K_{cat} 1580 min^{-1}; Anlezark et al 1995). Both compounds have been studied as prodrugs for ADEPT and GDEPT applications (Knox et al 1995; Friedlos et al 1997; Green et al 1997), although the requirement of this enzyme for a cofactor might make GDEPT applications more appropriate. The bystander properties of **1** have been shown to result from a cell-permeable metabolite (Bridgewater et al 1997).

The use of Enediyne Effectors in Prodrugs for GDEPT

The enediynes are a diverse class of natural products containing a conjugated double-triple-double bond (enediyne) (Nicolaou et al 1993). They are very potent cytotoxins, triggered by molecular rearrangements which juxtapose the conjugated triple bonds of the enediyne, initiating an electrocyclic reaction resulting in the formation of a

transient benzene 1,4-diradical. This can simultaneously abstract a proton (at C-4′ or C-5′) from a ribose group on each chain of duplex DNA, resulting in double strand breaks (De Voss et al 1990). Simplified versions of the natural products have lesser but still significant cytotoxicity (Nicolaou et al 1992). We recently reported the synthesis of the 4-nitrobenzyl carbamate analogue **4**, the first enediyne-based prodrug designed as a substrate for the *E. coli* aerobic nitroreductase enzyme. The 4-nitrobenzyl carbamate group is a good substrate for the enzyme, being reduced selectively by the bacterial enzyme and fragmenting on reduction to release amine-type effectors (Mauger et al 1994). In the presence of added enzyme the prodrug **4** was substantially (90-fold) activated in a cell-line assay (Hay et al 1995), suggesting its potential for ADEPT applications.

The use of Cyclopropylindole Effectors in Prodrugs for GDEPT

The cyclopropylindolines are another class of very potent anti-tumour antibiotics, exemplified by the natural product duocarmycin SA (**5**) and synthetic analogues such as CBI-TMI (**6**) (Boger & Johnson 1996). They are DNA-alkylating agents with a unique mode of action, forming adenine *N*-3 adducts in the minor groove, at adenine-thymine-rich sequences. The open-chain *seco*-phenol precursors of these compounds (e.g. **7**) retain the full cytotoxicity of the cyclopropyl products (Boger et al 1990), but offer little scope for prodrug chemistry because of their instability. For example, whereas carzelesin (**8**), an analogue in clinical trial, is formally a carbamate prodrug of a *seco*-phenol form, it is very labile, rapidly and non-specifically releasing the corresponding phenol in plasma (Li et al 1992).

We recently synthesized the first *seco*-amino compounds (**9** and **10**), in a search for analogues of similar cytotoxicity which could form more stable prodrugs through the amino group (Tercel et al 1996a; Atwell et al 1997). The amino-*seco*-CI-TMI derivative **8** reacts with DNA in a similar fashion to the corresponding oxygen analogues, with the only product isolated from alkylated DNA being an adenine *N*-3 adduct (Fan et al 1998). The cytotoxicity of the amino-*seco*-CI derivative **9** (IC50 320 nM in AA8 cells, 4 h exposure), although substantially less than that of the phenol analogue **7** (IC50 6 nM), is comparable with that of the most potent aniline mustards, whereas the amino-*seco*-CBI analogue (**10**) is a very potent cytotoxin (IC50 0·4 nM) (Atwell et al 1997).

The 6-nitro-*seco*-CI analogue **11** (an intermediate in one of the syntheses of **9**) was also shown to be a substrate for the *E. coli* nitroreductase with 400-fold-reduced IC50 against UV4 cells on addition of purified enzyme (Tercel et al 1996b). This suggests that 6-nitro-*seco*-CI compounds are a new class of potential prodrugs for the *E. coli* nitroreductase. Whereas the corresponding nitro-*seco*-CBI compound (**12**) is less selective (unpublished results, this laboratory), the very high potency of the amino analogue (**10**) makes it attractive as an effector for use with amine-modifying triggers.

Conclusions

Both ADEPT and GDEPT strategies are attractive options for improving the utility of systemic antiproliferative agents as anti-cancer drugs, although much work is still required. In the immediate future problems of immunogenicity in ADEPT and difficulties with the selective delivery and expression of genes in GDEPT will probably be the limiting factors, rather than the development of suitable prodrugs.

However, there is a need for prodrugs which diffuse efficiently through tumour tissue and which have optimised bystander effects (to compensate for the low efficiency of transfection in tumours). The recent development of a tissue culture model (multicellular layers) for investigating extravascular drug diffusion (Cowan et al 1996; Hicks et al 1997) is expected to facilitate studies of these aspects of prodrug design, which have thus far received little attention.

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