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Invited review

Prodrug strategies in cancer therapy

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Abstract – Systemic cytotoxic (anti-proliferative) anticancer drugs rely primarily for their therapeutic effect on cytokinetic differences between cancer and normal cells. One approach aimed at improving the selectivity of tumour cell killing by such compounds is the use of less toxic prodrug forms that can be selectively activated in tumour tissue (tumour-activated prodrugs; TAP). There are several mechanisms potentially exploitable for selective activation. Some utilise unique aspects of tumour physiology such as selective enzyme expression, hypoxia, and low extracellular pH. Others are based on tumour-specific delivery techniques, including activation of prodrugs by exogenous enzymes delivered to tumour cells via monoclonal antibodies (ADEPT), or generated in tumour cells from DNA constructs containing the corresponding gene (GDEPT). Because only a small proportion of the tumour cells may be competent to activate the prodrug, whichever activating mechanism is used, TAP need to be capable of killing activation-incompetent cells as well via a ''bystander effect'', in order to fully exploit these ''activator'' cells. A wide variety of chemistries have been explored for the selective activation of TAP. These include reduction of quinones, *N*-oxides, nitroaromatics and metal complexes by endogenous enzymes or radiation, amide cleavage by endogenous peptidases, and metabolism by a variety of exogenous enzymes, including phosphatases, kinases, amidases and glycosidases. © 2001 Éditions scientifiques et médicales Elsevier SAS

ADEPT / armed antibodies / GDEPT / hypoxia / peptide conjugates / tumour-activated prodrugs

1. Introduction

The majority of clinically-used anticancer drugs are systemic anti-proliferative agents (cytotoxins) that preferentially kill dividing cells, primarily by attacking their DNA at some level (synthesis, replication or processing). These cytotoxins have many advantages as anticancer drugs, especially the ability to kill large numbers of tumour cells with constant proportion kinetics [1]. However, these drugs are not truly selective for cancer cells, and their therapeutic efficacy is limited by the damage they also cause to proliferating normal cells such as those in the bone marrow and gut epithelia. This is particularly true in the treatment of solid tumours, where the majority of the tumour cells themselves are not dividing rapidly. One strategy aimed at providing substantial increases in the clinical efficacy of such drugs, especially against the more slowly-growing solid tumours, is the development of relatively non-toxic prodrug forms of these cytotoxins that can be selectively activated in tumour tissue.

Prodrugs can be defined as agents that are transformed after administration, either by metabolism or by spontaneous chemical breakdown, to form a pharmacologically active species. While prodrugs of cytotoxins have been used in cancer therapy for some time (e.g. araC esters [2] and cyclophosphamide [3]), these undergo activation non-specifically and are used primarily to modify drug uptake or pharmacokinetics by controlling physicochemical properties. Prodrugs of this type will not be discussed here. However, there is increasing interest and achievement in the development of tumour-activated prodrugs (TAP), and in the various ways these can be selectively activated in tumour tissue [4, 5]. This is a very large field, and cannot be covered in depth in a single review. The aim of the present article is to indicate the many approaches being taken to develop TAP, focusing

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particularly on the chemistry of the prodrugs and the mechanisms of selectivity, illustrating these with relevant examples.

2. Mechanisms of tumour-selective activation

2.1. Selective enzyme expression in tumour cells

While there is much evidence of varying enzyme regulation in tumours, consistent patterns of expression are elusive. Consequently, attempts to develop drugs targeted to "tumour-specific" enzymes in general have not been very successful. An early example was the 5-aziridinyl-2,4-dinitrobenzamide CB 1954 (**1**), which could effect complete cures of the Walker 256 rat carcinoma. This tumour was found to express a high level of the enzyme DT diaphorase [6], which activated the prodrug through reduction of the 4-nitro group [7] (see Section 8.1.4). However **1** was a much poorer substrate for human than rat DT diaphorase ($K_{cat} = 0.64$ min^{-1} compared with 4.1 min^{-1}) [8]. The aziridoquinone EO9 (**2**) is also specifically activated by DT diaphorase [9], and its cytotoxicities in a range of tumour lines in culture correlate well with the cellular levels of DT diaphorase [10]. However, clinical results with EO9 to date have again been disappointing [11, 12], and have been attributed to varying activity levels of the enzyme in clinical tumours [13], as well as to a short halflife [14]. Prodrugs of this type will not be discussed here.

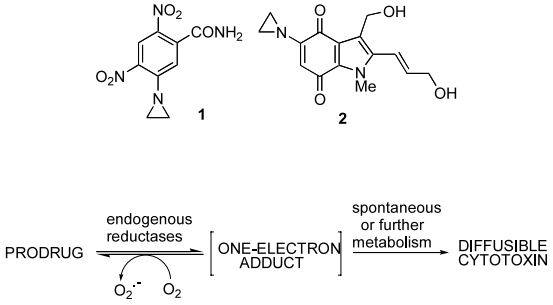


Figure 1. Mechanism of prodrug activation under hypoxia.

Figure 2. Mechanism of prodrug activation by therapeutic radiation.

2.2. Tumour hypoxia

It is now well-known that the imperfect neovascularisation seen in growing solid tumours results in limited and inefficient blood vessel networks, and restricted and often chaotic blood flow [15]. This and the high and variable interstitial pressures caused by the growing tumour [16] lead to the presence of a variable but significant proportion of hypoxic cells [17]. Such hypoxia can be classified into two broad types. Chronic or diffusion hypoxia keeps cells sufficiently distant from the nearest blood capillary hypoxic for long periods, whereas transient or perfusion hypoxia results from the temporary shut down of blood vessels, placing sections of tissue under hypoxia for shorter periods [18]. Hypoxia appears to be a common and unique property of cells in solid tumours, and is an important potential mechanism for the tumour-specific activation of prodrugs. Since the enzymes generally exploited to activate hypoxia-selective TAP (e.g. hP450R, xanthine oxidase) [19] occur in all cells, discrimination between tumour and normal tissue is usually achieved by initial reduction of the trigger to a transient intermediate able to be efficiently back-oxidised by molecular oxygen (*figure 1*).

2.3. Therapeutic radiation

In principle, the reducing species produced from the radiolysis of water by ionising radiation can be used to activate prodrugs in an oxygen-inhibitable manner, restricting activation to the hypoxic cells in the radiation field and avoiding the use of endogenous enzymes for activation [20] (*figure 2*). Radiation therapy is widely used in cancer treatment, and modern equipment can deliver radiation dose very selectively to solid tumours. One difficulty in designing prodrugs for this approach is the small amount of reducing equivalents delivered by a therapeutic dose of radiation, requiring the release of very potent active species [4, 20].

2.4. pH differences

Another consequence of the limited blood flow seen in many solid tumours is a generally lower extracellular pH [21]. This is considered to be caused by the inefficient clearance of metabolic acids from chronically hypoxic cells, a phenomenon that can lower 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). Cell-excluded prodrugs that can be selectively

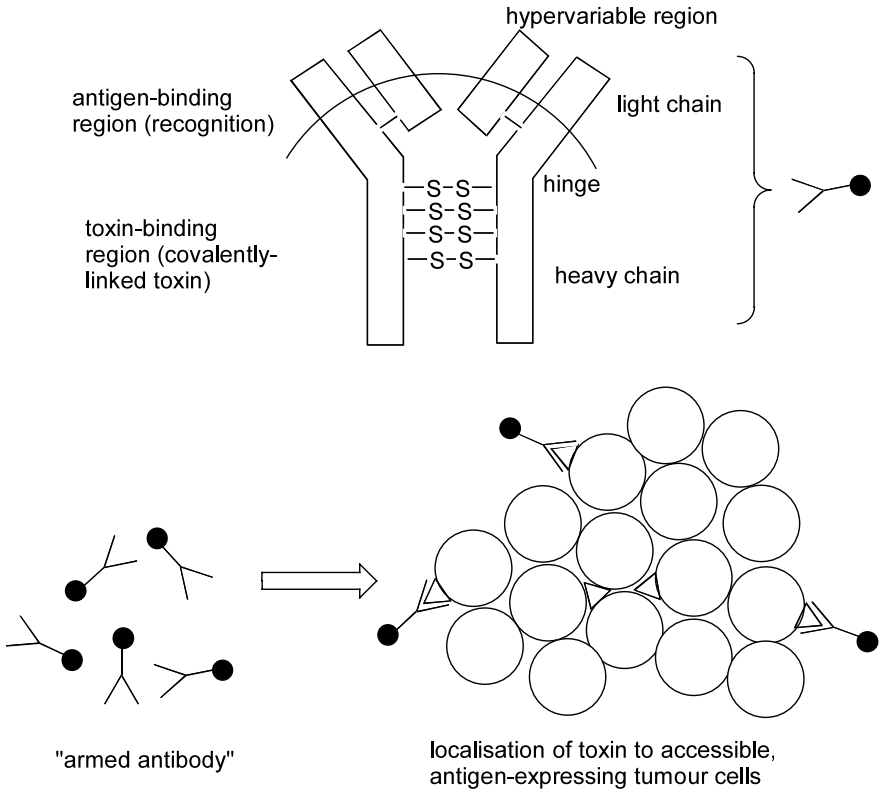


Figure 3. Construction and mechanism of antibodies 'armed' with small-molecule cytotoxins.

activated by this lower extracellular pH in solid tumour tissue have been described [22], but the concept has not received much attention, and will not be discussed here.

2.5. Tumour-specific antigens

It has long been known that certain types of tumour cells present characteristic tumour-associated antigens on their surface [23]. The hypothesis was that conjugation of toxic drugs to the associated antibodies would deactivate the drug (by limiting diffusional access to cells) without changing the selectivity of binding of the antibody, allowing it to locate on (antigen-bearing) tumour cells, internalise and release toxin (*figure 3*). This type of 'armed antibody' approach has not been particularly successful until recently. One likely reason is that this method delivers an extremely small proportion of the toxin to the interior of the target tumour cells, because of difficulties in distribution of these large constructs. In addition, in heterogenous solid tumours, many cells may express low or no levels of the target antigen.

Antibody-directed enzyme-prodrug therapy (ADEPT) is an adaption of the immunotoxin concept, where instead of the toxin being localised on tumours by attachment to the antibody, an exogenous (non-human) enzyme is attached and so localised [24] (*figure 4*). A prodrug that is a good substrate of the exogenous (but not of any human) enzyme is then given, and is catalytically activated only in the vicinity of the tumour cells. If the released cytotoxin has the appropriate properties to have a good bystander effect (able to diffuse from the cell where it is generated to enter and kill surrounding

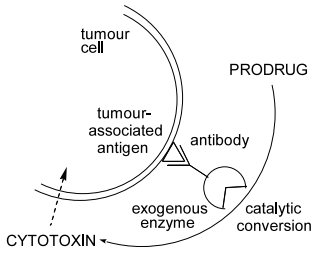


Figure 4. Antibody-directed enzyme-prodrug.

tumour cells that may not possess prodrug activating ability), this can overcome the problems of limited access of the conjugate and heterogenous expression of the antigen. A further increase in efficacy can be achieved if the prodrug is designed to be excluded from cells until it is activated.

2.6. Excreted tumour-specific enzymes

This class of prodrugs comprise toxins attached to small peptides to form 1:1 conjugates. The peptide prodrugs are significantly excluded from cells. These peptides are designed to be cleaved specifically by enzymes known to be produced and secreted preferentially by tumour cells (e.g. PSA from prostate tumours).

2.7. Gene-directed enzyme-prodrug therapy (GDEPT)

ADEPT solves some of the distribution and accessibility problems of immunotoxins, by delivering an enzyme rather than a toxin specifically to tumour cells. It is limited to the use of enzymes that do not require energy-producing cofactors (unless these are delivered as well), and has the likelihood of generating immune responses to the foreign proteins used. An alternative concept is gene-directed enzyme prodrug therapy (GDEPT), where the enzyme is targeted to tumour cells by integrating the gene which produces it into the genome of the tumour cells. This theoretically retains the advantages of ADEPT in terms of selective and sufficient access of the activated drug to tumour cells, and expands the class of available enzymes to those that require endogenous cofactors. However one approach to design in selectivity between prodrug and effector is lost, since the prodrugs must be able to enter cells freely. This also has implications for prodrug distribution, with the need to avoid those likely to bind to cellular macromolecules.

3. General concepts for the design of tumour-activated prodrugs

As outlined above, TAP must fulfil a number of criteria. They must distribute efficiently to the remote (hypoxic) regions in tumours, and undergo selective cellular metabolism in tumours to generate a cytotoxic species capable of diffusing a limited distance to kill surrounding tumour cells that may lack the ability to activate the prodrug. Regardless of the mechanism of their selective activation, it is useful to consider TAP as comprised of three distinct (although possibly not separate) domains: trigger and effector units joined by a linker mechanism [4, 25] (*figure 5*). The role of the trigger unit is to undergo efficient metabolism by one of the above tumour-specific mechanisms. The role of the linker is to deactivate the prodrug until trigger metabolism, then rapidly transmit the change to the effector in such a way as to cause rapid and substantial activation. The role of the effector is to kill cells rapidly and under all conditions of pH and cell cycle status, and to be capable of diffusing a sufficient distance to cause a substantial bystander effect. This requires both a suitable half life (suggested to be from many seconds to a few minutes), and generally precludes effectors that bind tightly to macromolecules such as DNA [26]. This general concept highlights the potential for the modular design of TAP, with both trigger and effector units optimised for their individual roles, and provides some general criteria for the design of these components.

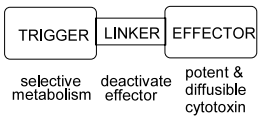


Figure 5. Trigger-linker-effector concept for prodrug design.

4. TAP activated by tumour hypoxia

4.1. Introduction

The rates of both one-electron reduction to the initial one-electron species (*figure 1*), and the scavenging of this with molecular oxygen in normal cells, are largely controlled by the size of the redox couples involved. Thus the one-electron reduction potential of the trigger unit is an important design parameter for hypoxia-selective TAP. Another important consideration is that hypoxic cells are usually out of cycle due to the lack of oxygen and other nutrients, which makes them more resistant to most cell cycle specific "antiproliferative" drugs that preferentially attack cycling cells. This puts constraints on the design of the effector unit, with the less cell cycle specific toxins such as DNA alkylating or direct DNA breaking agents preferred. Hypoxic cells are also usually also the most remote from blood vessels,

making them difficult to reach with high molecular weight prodrugs [18].

Finally, because only a small proportion of the cells in a solid tumour are likely to be hypoxic at any one moment, prodrugs that kill only hypoxic cells appear unlikely to be curative on their own. However, because of transient hypoxia [15], a significant fraction of solid tumour cells may eventually spend some time in a hypoxic state, so that fractionated treatment could theoretically cause substantial killing [27]. Another approach is to actively exploit the small hypoxic cell population to kill the surrounding more oxygenated cells in the tumour as well [26]. This uses prodrugs that are converted into (or release) much more cytotoxic species that can diffuse a limited distance. Exploitation of this 'bystander effect' uses the unique hypoxic cells in tumours to enhance the killing effect of the prodrug, making single-agent therapy possible. The major classes of triggers used for hypoxia-selective TAP have been aromatic and aliphatic *N*-oxides, quinones, aromatic nitro groups, and cobalt complexes.

4.2. Aromatic N-oxides

The best-known of this class of compounds is tirapazamine (3-aminobenzotriazine-1,4-di-*N*-oxide (**3**) (*figure 6*)), which is likely to be the first clinically-useful hypoxia-selective TAP [28]. Tirapazamine undergoes ready enzymic one-electron reduction to form an intermediate oxidising radical (*figure 6*), usually shown as a nitroxide radical [29] (although it is also considered to be a carbon-centred species; **4**), and ultimately the two-electron product **5**. The radicals cause breaks at the C-4′ ribose site of DNA, followed by the oxidation of these by (among other things) tirapazamine itself [30], via formation of a covalent adduct formed by reaction at the *N*-oxide oxygen. The DNA breaks generated by tirapazamine correlate well with its cytotoxicity [31]. The enzymes primarily responsible for the hypoxia-selective cytotoxic metabolism of **3** are cytochrome P450

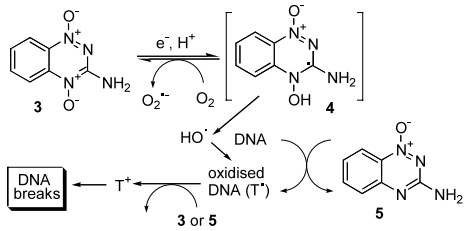


Figure 6. Metabolism of tirapazamine.

and cytochrome P450 reductase [19], although it is also reduced under hypoxia by aldehyde oxidase, xanthine oxidase [32] and nitric oxide synthase [33]. Although **3** forms a monofunctional radical species, it generates a high proportion of double-strand DNA breaks, possibly by high local radical concentrations generated by an undefined intranuclear reductase associated with DNA [28].

Tirapazamine shows high hypoxic selectivity (100–200-fold) in cell suspension cultures, but its diffusion through tissue is limited by its metabolism to the (non-diffusible) radical species [34]. Hypoxic cell killing extends over a much wider range of oxygen concentrations (as high as 2% O_2) [35] than with most bioreductive drugs, so that activation is not restricted to completely anoxic tissues [36]. Tirapazamine has had extensive clinical trials, both as a single agent [37] and in combinations with radiation [38]. Ongoing Phase II trials in conjunction with radiation (to kill oxygenated cells) in head and neck cancer are reported as encouraging [39]. It also enhances the effects of cisplatin, probably by delaying the repair of cisplatin-induced DNA cross-links in hypoxic cells [40]. Superior response rates compared to cisplatin alone have been shown in cervical cancer [41], mesothelioma [42], malignant melanoma [43] and particularly non-small-cell lung cancer [44]. Clinical toxicities include ototoxicity and muscle cramping [45]. A laboratory study showed that **3** caused time and dose-dependent retinal damage in mice [46], but this does not appear to be a clinical issue.

Related quinoxalinecarbonitrile 1,4-di-*N*-oxides are also potent and highly selective hypoxia-selective TAP [47]. These compounds replace the 2-nitrogen in the benzotriazine unit of **3** with a C–CN unit. Structure–activity studies show that hypoxic selectivity is retained when the 3-amino group is replaced by H or NHR, but not when replaced by Cl. Soluble analogues such as **6** retain potency and hypoxic selectivity [48]. Some of these compounds show activity in anti-mycobacterial [49] and anti-tuberculosis [50] in vitro screens, although it is not known whether this is related to their hypoxic selective activity. A series of 1,2,5-oxadiazole *N*-oxides (e.g. **7**) have also been reported [51], but do not have significant hypoxic selectivity.

Imidazo[1,2-*a*]quinoxaline *N*-oxides such as **8** (RB 90740) showed hypoxic selectivity in cell culture [52]. They are activated through reduction by P450 reductase and cytochrome *b*5 reductase, although no clear relation between levels of these enzymes and hypoxic toxicity was seen [53]. RB 90740 was shown to cause DNA

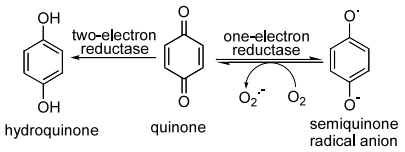
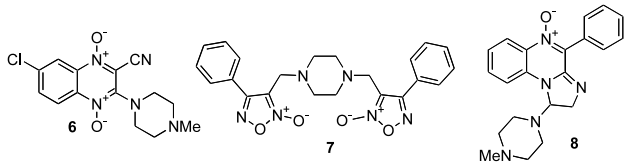


Figure 7. Quinone metabolism.

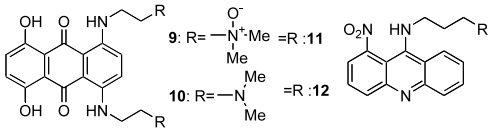
strand breaks under hypoxic conditions, but was not cytotoxic to hypoxic cells in murine tumours in vivo. This was suggested due to the very low levels of hypoxia (<0.02% oxygen) needed to produce toxicity in vitro [54].



4.3. Aliphatic N-oxides

Polycyclic DNA binding agents bearing cationic tertiary amine side chains are potent cytotoxins, and are among the most widely used anticancer drugs. The cationic side chains ensure good uptake into cells and tight binding to DNA, thus interfering with topoisomerase enzyme function. The loss of cationic properties following *N*-oxidation of these side chains greatly decreases DNA binding affinity and thus cytotoxicity [55]. While the enzymic reduction of aliphatic *N*-oxides to the corresponding tertiary aliphatic amines, primarily by the CYP3A isozyme of NADPH:cytochrome C (P-450) reductase [56], is not a one-electron process, it is inhibited by oxygen, probably because of direct competition between oxygen and the drug at the enzyme site. Thus aliphatic *N*-oxides of this type have been explored as hypoxia-selective TAP. They are best represented by AQ4N (**9**), where reduction of the *N*-oxides generates the tight DNA binding agent and topoisomerase II inhibitor AQ4 (**10**) [57]. Combination studies of AQ4N with radiation therapy in a series of murine solid tumours in vivo showed that the drug was not significantly active as a single agent, but potentiated the effects of radiation (which kills the oxygenated tumour cells) in a dose-dependent manner [58]. Increasing the hypoxic fraction in the tumours by clamping or by treatment with hydralazine also gave substantial enhancement of anti-tumour effect. Combination of AQ4N with cyclophosphamide in murine tumour models also gave increased efficacy, in some cases superior to that of similar combinations of tirapazamine and cyclophosphamide [59]. AQ4N is due to begin Phase I clinical trials shortly.

N-Oxide derivatives of other cationic DNA-intercalating agents have also been shown to have hypoxia-selective activity [60], suggesting this is a general design concept. A particularly interesting case is nitracrine *N*-oxide (**11**). Nitracrine itself (**12**) is a very potent cytotoxin with moderate hypoxic selectivity, through reductive activation of the nitro group (see Section 4.5.1). *N*-Oxidation of the cationic side chain to give **11** results in a much lower DNA binding constant [61], better distributive properties as demonstrated by studies with EMT6 spheroids [62], and exceptional hypoxic selectivity (>1000-fold in cell culture) [61]. This is attributed to **11** being a 'bis-bioreductive' compound, requiring two independent reduction steps (nitro and *N*-oxide) for full activation, with the *N*-oxide demasking needing to occur before nitro reduction [63]. However, **11** still had low extravascular diffusion rates and little activity in vivo against the hypoxic sub-fraction of cells in KHT tumours [62]. Since this was considered to be due to too-rapid metabolism, modulation of the rate of metabolism of both the nitro group (by lowering its reduction potential through chromophore substitution [62]) and the aliphatic *N*-oxide (by changing its steric environment [64]) were studied, but neither approach resulted in significant improvements of in vivo activity.



4.4. Quinones

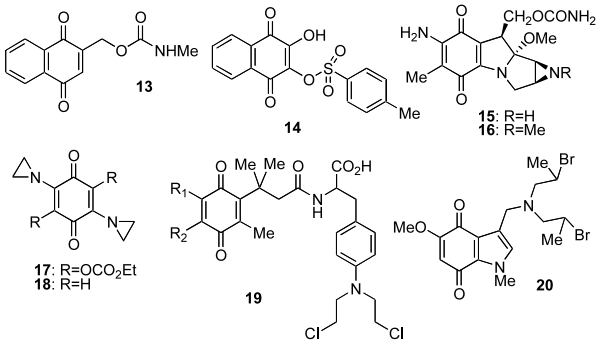
Quinone analogues were among the first compounds explored as hypoxia-selective TAP. They are known to undergo one-electron reduction by cytochrome C P-450 reductase to the semiquinone radical anion [65] that can be back-oxidised by molecular oxygen in normal well-perfused cells (*figure 7*). The earliest studies explored quinones (e.g. **13**) with incipient leaving groups at the 2-position, in order to generate reactive quinone methide intermediates [66]. These, and more recent related analogues (e.g. **14**) [67], have low hypoxic selectivity.

The first clinical agent designated as a hypoxia-selective TAP was the natural product mitomycin C (**15**) [68],

but even this shows only marginal hypoxic selectivity, which is not the main basis of its usefulness. The analogue porfiromycin (**16**) shows higher selectivity, and has been clinically evaluated as a hypoxia-selective TAP [69]. In the case of mitomycin, porfiromycin and analogues, generation of cytotoxicity following reduction is by subsequent well-defined fragmentation to species that cross-link DNA via guanine–guanine crosslinks in the major groove [70]. A clinical study of porfiromycin and radiation in head-and-neck cancer concluded it was useful as an adjunct to radiation therapy [71]. However, a potential drawback to quinone triggers for TAP is that they are also often good substrates for two-electron reductases, particularly DT diaphorase (DTD; NQO1; NAD(P)H: quinone-acceptor oxidoreductase) [72].

In fact the aziridinylquinones, represented by diaziquone (AZQ) (**17**), are simpler analogues of the mitomycins that appear to be primarily activated by DTD [73]. In a series of AZQ analogues, there was a correlation between the rate of reduction by DTD and the cytotoxicity of the compounds in HT-29 cells [74]. The related DZQ (**18**) was shown by ligation-mediated PCR to alkylate DNA at 5′-(A/T)G(C)-3′ and 5′-(A/T)A-3′ sequences in both DTD-proficient (HT-29) and DTD-deficient (BE) cell lines, suggesting that enzymes other than DTD can also effect reduction [75]. Because of this propensity of quinones to be reduced by DTD, an enzyme that is over-expressed in many tumours, much work has been done to try and utilise this to selectively activate quinone-based drugs in tumours. The best example of this is the indoloquinone EO9 (**2**) but, as discussed in Section 2.1, this approach has not been particularly successful.

Quinones have also been used as triggers for the physical release of effectors [76]. Examples are compounds such as **19**, which expels melphalan as the effector by cyclolactonisation following reduction [77], and **20**, where reduction is followed by direct C–N bond cleavage to release an aliphatic mustard [78].



4.5. Nitroaromatics

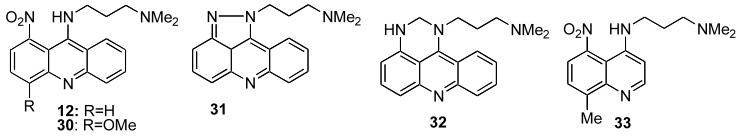
These have been extensively studied, and have been used to define and characterise many of the design concepts for hypoxia-selective TAP [4]. Despite this extensive work, no nitroaromatics have been used clinically primarily as TAP.

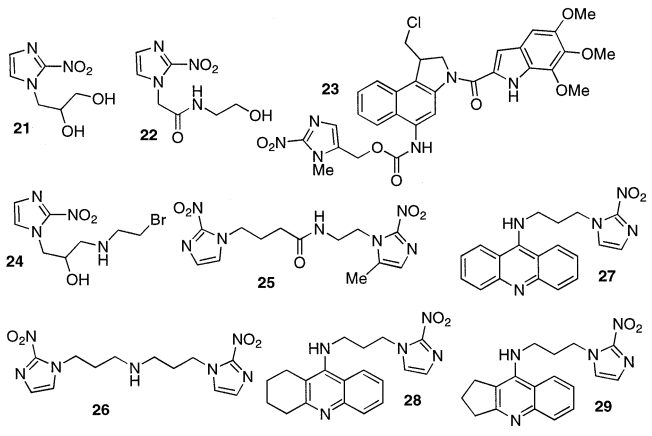
4.5.1. Activation by uncontrolled fragmentation

Nitroaromatic compounds are reduced in cells by a number of flavoprotein enzymes, which effect stepwise addition of up to six electrons, but the major enzymic metabolite is usually the four-electron species (hydroxylamine). For compounds of appropriate reduction potentials (suggested to be in the range −330 to −450 mV) [79], the first nitro radical anion one-electron adduct can be scavenged efficiently by molecular oxygen, restricting activation to hypoxic cells [80]. Hypoxia-selective TAP were developed from the early nitroimidazole-based oxygen-mimetic radiosensitisers such as misonidazole (**21**) and etanidazole (**22**). These are metabolised selectively under hypoxia to give ill-defined DNA-alkylating species, but are only weakly cytotoxic, and with moderate (ca. 10-fold) hypoxic cell selectivity in tissue culture [81]. More potent nitroimidazole-based compounds such as **23** also show only moderate hypoxic selectivity (15–40-fold) in cell culture [82]. In order to increase absolute potency, nitroimidazole analogues bearing an alkylating unit were studied. The bromoethyl analogue CI-1010 (**24**; *R*-enantiomer) proved not only more potent than simple nitroimidazoles, but also had higher hypoxic selectivity (ca. 100-fold), due to the generation of DNA crosslinking species under hypoxia [83]. However, this drug causes irreversible retinal damage [46, 84], and was not used clinically.

In a logical step from this, bis(nitroimidazoles) were explored, in the expectation of providing potential DNA crosslinking species only on double bioreductive activation [85]. However, while some of these compounds (e.g. **25**) showed high hypoxic selectivity (>200-fold in AA8 cells) they were not DNA cross-linking agents. They produced fewer DNA single-strand breaks than mononitroimidazoles at equivalent toxicity, suggesting the formation of some type of duplex DNA lesion (locally doubly damaged sites) [86]. Analogues (e.g. **26**) with cationic linkers were the most cytotoxic, with rapid kinetics of killing under hypoxia resulting in high hypoxic selectivity at early times in cell culture [87]. Ni-

troimidazoles have also been tethered to DNA targeting species in attempts to increase potency. Compounds such as NLA-1 (**27**) are more potent hypoxia-selective agents than simple nitroimidazoles [88, 89], but later analogues such as THNLA-1 (**28**) [90] and NLCPQ-1 (**29**) [91] have been developed primarily as chemopotentiating agents of hypoxic cells.





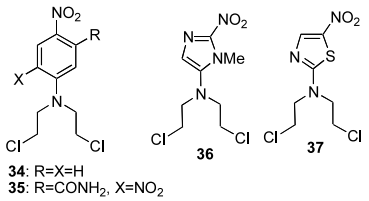
The 1-nitroacridine derivative nitracrine (**12**) is a much more potent hypoxia-selective TAP than the imidazoles, likely due to its tight DNA binding. Nitracrine shows consistent hypoxic selectivity in cell culture [92], but not against hypoxic cells in solid tumours, likely because of rapid metabolism due to its high reduction potential (−303 mV) [93] and tight DNA binding [92] that slows diffusion into hypoxic areas [94]. 4-Substituted analogues with lower redox potentials (such as **30**) had improved metabolic stability and some activity against hypoxic cells in vivo, but extravascular transport limitations were still limiting [95]. Nitracrine is known to alkylate DNA following reduction by thiols or enzymes, but the nature of the metabolites are still debated. Recent studies [96, 97] have failed to trap the putative 1-hydroxylamine, but showed a range of metabolites, including **31** and **32**.

The related 5-nitroquinolines, while less potent, showed higher hypoxic selectivity in cell culture. Despite 10-fold lower DNA binding constants, analogues such as **33** were also not selectively active against hypoxic cells in vivo, probably due to rapid metabolism [98]. The low *K*-value (the oxygen concentration for cytotoxic potency equal to the mean of the potencies at zero and infinite oxygen) for **33** of 0.02% oxygen [99] is also a limiting factor for in vivo activity.

4.5.2. Activation by electron release to a pre-positioned effector

Enzymic reduction of a nitro group to a hydroxylamine produces a very large change in electron distribution, as indicated by the σ change of 1.12 Hammett units for *para* substituents in a benzene ring. Since the stability and alkylating reactivity of aromatic nitrogen mustards is determined almost entirely by the electron density on the mustard nitrogen [100], this change can result in a very large increase in potency. The first nitrobenzene compound shown to possess hypoxic selectivity was the dinitroaziridine CB 1954 (**1**). This shows a modest selectivity of about 3.5-fold in hypoxic UV4 cells in culture [101]. The simplest such prodrug, the mustard **34**, also has modest hypoxic selectivity, despite a low nitro group reduction potential of about −510 mV, which suggests that only a small proportion of the drug is likely to be metabolised [102]. Derivatives with higher nitro group reduction potentials (comparable to that of **1**), such as the 2,4-dinitro analogue **35**, had hypoxic selectivities of 60–70-fold for hypoxic UV4 cells in culture [101], as did a number of other isomers [103]. The probable reason for the higher selectivity of **35** and analogues is that the mustards are much less efficient substrates than **1** for the major aerobic nitroreductase (NTR) DTD [101].

However, compounds such as **35** are a compromise. To ensure the nitrobenzene unit has a high enough reduction potential to be a good substrate, it must be made very electron-deficient, resulting in agents of relatively low cytotoxicity even after reductive activation. Analogues based on nitroheterocyles of higher intrinsic reduction potentials have been prepared (e.g. **36**, **37**) [104, 105], but did not show significantly greater hypoxic selectivity.



4.5.3. Activation by controlled fragmentation to release an effector

This has been the most widely used mechanism to generate the effector from hypoxia-selective TAP following bioreduction. Nitrobenzyl carbamates undergo multi-electron reduction to the hydroxylamine or amine species, which then fragments to generate a quinoneimine methide and an amine [106] (*figure 8*). This exemplifies well the trigger-linker-effector concept for TAP (see *figure 5*). However, the reduction potentials of the benzene-based compounds are too low for efficient reduction by cellular NTRs; for example, a study of 5-fluorouracil prodrugs of this type (e.g. **38**) showed no hypoxic selectivity in cells [107]. This trigger system is of more interest in GDEPT applications (see Section 8.1.4).

Prodrugs such as **39** were designed to generate effectors by a 'through-space' cyclisation–extrusion process [108]. The geometry of the compound proved important, with preorganisation of the molecule in the correct conformation enhancing the rates of cyclisation. Radiolytic reduction studies [109] suggested that cyclisation probably occurred via the hydroxylamines. However, the reduction potentials of the compounds were too low for efficient cellular reduction. The 2,6-dinitrophenyl derivative (**40**) has a higher reduction potential, and the rates of cyclisation/extrusion of the mono-hydroxylamine reduction product is greatly accelerated by the presence of an H-bonding "conformational lock" between the anilino NH group and the adjacent 2-nitro group [110]. This compound did not show significant hypoxic selectivity, but cell killing by radiation-induced reduction was demonstrated [110].

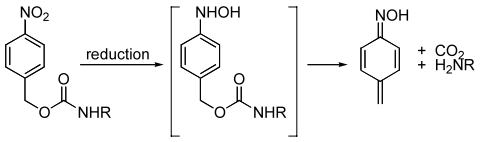
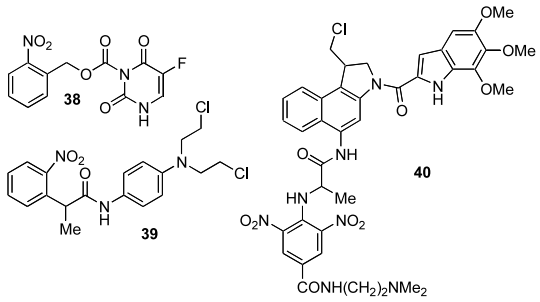
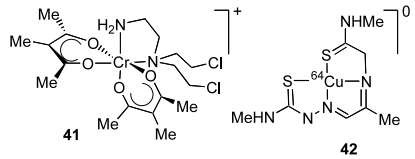


Figure 8. Carbamate fragmentation.



4.6. Metal complexes

Nitrogen-based complexes of many transition metals such as cobalt are very stable in the low-spin Co(III) oxidation state. They have reduction potentials in the appropriate range to undergo reduction by cellular reductases, but this is inhibited in oxygenated cells apparently by competition for cellular reductants between the Co(III) complex and oxygen [111]. However, under hypoxia the unstable high-spin Co(II) complex resulting from reduction rapidly releases its ligands to form the very stable hexaaquo Co(II) species. Because nitrogen mustards are much more stable when metal-bound than when free, complexes such as **41** demonstrate modest hypoxia-selectivity in cell culture [112]. This compound had a K value (ca. 0.02% O_2) similar to those seen for quinones and nitroaromatics [113], but showed little activity against hypoxic cells in vivo, and has not been developed further. In a different approach, radio-labelled ([64]Cu) bis(thiosemicarbazone) and bis(salicylaldimine) copper complexes (e.g. **42**), based on the perfusion tracer CuPTSM, were shown to have hypoxic selectivity in Chinese hamster ovary cells in culture [114].



5. TAP activated by therapeutic radiation

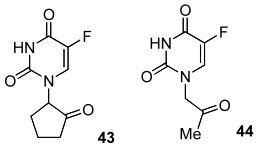
5.1. Introduction

Prodrugs that could be activated by the reducing equivalents, primarily the hydrated electron, generated by the irradiation of water in cells during radiotherapy have been described recently (*figure 2*). Such compounds can potentially achieve a double degree of selectivity,

being activated only in hypoxic regions within the radiation field, since the hydrated electron is very short-lived, and both it and the initial one-electron adducts of the prodrugs are rapidly scavenged by molecular oxygen [115, 116]. Because these compounds do not rely on enzymes for their bioreduction, activation can occur in all hypoxic regions of tumours including areas of necrosis, and oxygen-insensitive reduction by two-electron reductases can be avoided. However, this concept places considerable demands on the design of the prodrugs. Because of the small yield of reducing equivalents generated by a therapeutic dose of radiation (about 0.6 μmol kg^{-1} from a 2 Gy dose) [115], both very efficient triggers and very potent effectors are required. Two general types of radiation-activated prodrugs have been reported.

5.2. Prodrugs of 5-fluorouracil

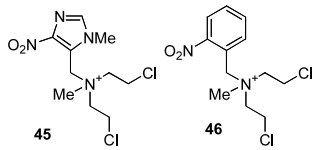
Following on from earlier work [117] on a 5-fluorouracil dimer, Nishmoto et al. have reported a series of related (oxocycloalkyl)uracil derivatives (e.g. **43**) that undergo one-electron reduction by hydrated electrons on irradiation in anoxic aqueous solution, followed by C(1′)–N(1) bond cleavage to release 5-fluorouracil in high yield [118]. An open-chain derivative (**44**; OFU001) showed similar *G* values (mols converted by 1 J of absorbed radiation energy) for decomposition under both hypoxic and aerobic conditions (ca. 0.3 μmol J^{-1}). However, there was a significant difference in *G* values for release of 5-fluorouracil (0.19 μmol J^{-1} under hypoxia but only 0.01 μmol J^{-1} under aerobic conditions) [116]. In vivo studies of **44** in mice bearing sub-cutaneous SCCVII tumour showed that 5-fluorouracil was released in the tumours in amounts dependent on the radiation dose (179 ng g^{-1} at 30 Gy), but no significant therapeutic effects were seen [119].



43 44

5.3. Aromatic quaternary salts

A series of quaternary ammonium salts of mechlorethamine have been reported as model radiation-activated prodrugs [115]. These are very efficient at deactivating the cytotoxic amine effector, and can be classified into two types according the stoichiometry of effector release [120]. Type I agents (e.g. **45**) release effector with one reducing equivalent via a benzyl-type radical and the concomitant generation of products from benzyl radical dimerisation. Type II agents (e.g. **46**) require multiple reducing equivalents to release the effector, and do not produce radical dimerisation products. Type I triggers are preferable for the development of radiation-activated prodrugs bearing more potent effectors [120].



45 46

6. TAP activated by localisation to tumour-specific antigens

6.1. Armed antibodies (immunotoxins)

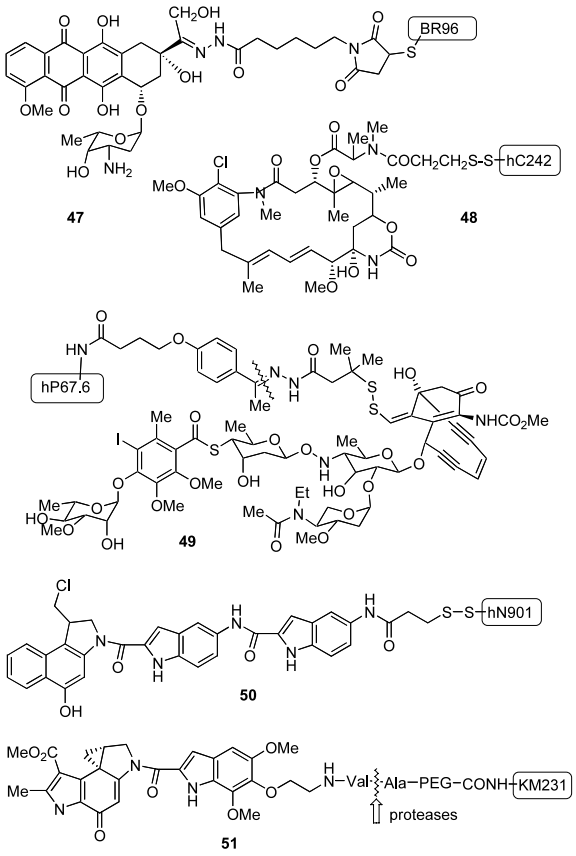
As noted in Section 2.5, recent advances have led to a resurgence of interest in tumour-specific antibodies armed with very potent small-molecule cytotoxins. More specific coupling reagents allow the preparation of well-defined conjugates, where a number of toxin molecules are linked to antibodies by linkers designed to be cleaved following uptake of the conjugates by endocytosis. Doxorubicin continues to be a toxin of choice for arming antibodies. The most well-studied of these is perhaps the BR96-DOX conjugate (**47**), where an average of eight molecules of doxorubicin are linked via an acid-labile hydrazone link through the C-14 carbonyl to the chimeric mAb BR96, which binds to a modified Le^y antigen on tumour cells. In vitro studies [121] show that the major route of breakdown of **47** in vitro is acid-catalysed hydrazone hydrolysis. BR96-DOX produced cures of established sub-cutaneous RCA human colon carcinomas in athymic mice and rats [122], where maximum tolerated doses for free doxorubicin were ineffective. A recent Phase I clinical trial of **47** in 66 patients with metastatic colon and breast cancers that expressed the Le^y antigen determined the optimal dose to be 700 mg m^{-2} (equivalent to 19 mg m^{-2} of doxorubicin). Toxicity was mild and dissimilar to that of doxorubicin, and the authors concluded that phase II study in doxorubicin-sensitive tumours was warranted [123].

The very potent maytansinoid-type tubulin inhibitors are also being used for arming antibodies [124]. For example, **48** (SB 408075, C242-DM1) comprises an aver-

age of four molecules of the very potent synthetic maytansinoid DM1 attached by a disulphide link to hC242, an antibody to a mucin-like glycoprotein on colorectal cancer cells. This conjugate effected cures in mice bearing large COLO205 human colon tumour xenografts [125], and is in Phase II trials.

The extremely potent calicheamicin and benzindoline type toxins are of particular interest in this role. The DNA-cleaving agent calicheamicin and its analogues have been used for some time to arm antibodies [126, 127], and **49** (gemtuzumab ozogamicin, CMA-676, Mylotarg) was the first antibody armed with a small-molecule cytotoxin to reach clinical trial. This has an average of four to five calicheamicin molecules linked via a sterically hindered disulphide, through an acid-labile hydrazone linker, to a humanised hP67.6 IG1-based antibody. This recognises the CD33 antigen that occurs on normal and leukaemic myeloid progenitor cells, but not on haematopoietic stem cells [128]. It shows very high selectivity for CD33-positive cells, but is susceptible to P-glycoprotein-mediated multidrug resistance [129]. In Phase II studies in relapsed AML patients, an overall 30% response rate was seen [130]. The conjugate has also been reported to be active clinically in brc/abl-positive CML [131].

The class of very cytotoxic DNA minor groove alkylators exemplified [132] by the natural product CC-1065 has also been used to arm antibodies. The conjugate **50** (hN901-DC1) comprises an average of 3.3 adozelesin-type toxin molecules linked via a disulphide to the hN901 antibody that targets the CD56 tumour-associated antigen [133]. The toxins are not chemically deactivated by the linkage, but are substantially less toxic when conjugated. The conjugate **51** (KM231-DU257) contains an average of two molecules of the duocarmycin analogue DU257, linked via a PEGylated dipeptide (HO_2C-Val-Ala-NH_2) to an M231 antibody that targets the sLe^a antigen [134]. The PEGylated linker prolongs plasma halflife, and the Val-Ala link is cleaved by tumour proteases to release primarily the DU257-Val conjugate, that has similar potency to DU257 itself.



6.2. Antibody-directed enzyme-prodrug therapy (ADEPT)

This is an adaption of the immunotoxin concept, delivering an enzyme rather than a toxin to antigenic sites on tumour cells. The tethered enzyme then catalytically metabolises a separately-given prodrug, releasing an effector able to diffuse to and enter surrounding tumour cells [23, 24]. The efficacy of this approach depends on a number of factors related to choice of antibody, enzyme and prodrug. Particular requirements of the prodrug include the ability to be excluded from cells (usually achieved by high hydrophilicity and/or possession of a negative charge) until activation, followed by release of a diffusible effector with a substantial bystander effect.

6.2.1. Prodrugs for phosphatase enzymes

Some of the earliest ADEPT prodrugs evaluated were phosphates, since both aromatic (e.g. **52**; etoposide phosphate) [135] and aliphatic (e.g. **53**; mitomycin phosphate) [136] phosphates appear to be efficiently cleaved by endogenous alkaline phosphatases. However, the abundance of phosphatases in human serum and other tissues has meant that interest in phosphates as prodrugs has focused primarily on their direct use, rather than in ADEPT protocols. The antivascular agent **54** (combretastatin phosphate) is also being used in this way [137].

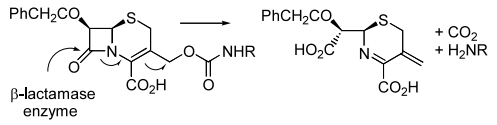
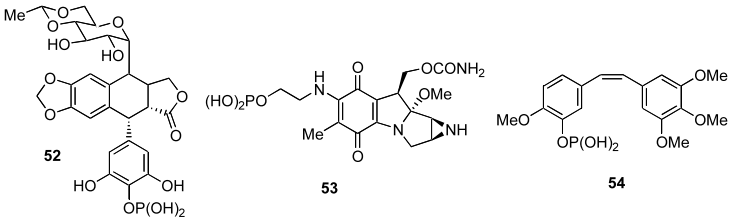
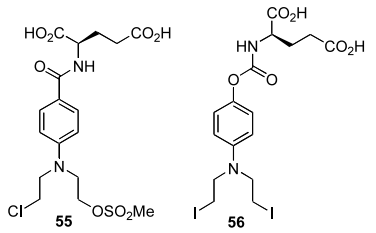


Figure 9. Fragmentation of β-lactamase prodrugs.



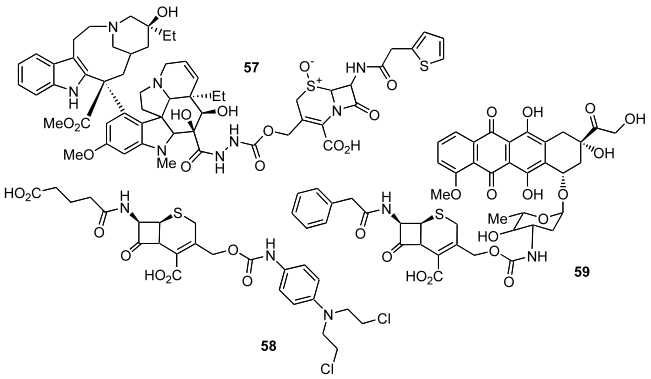
6.2.2. Prodrugs for peptidase enzymes

Considerable work has been done with glutamate-type prodrugs of mustards that are substrates for the *Pseudomonas*-derived enzyme carboxypeptidase G2 [138]. These prodrugs (e.g. **55**, **56**) are effectively excluded from cells by the diacid side chain, with cleavage of the glutamate-like amide or carbamate bond simultaneously releasing a more lipophilic effector and activating the mustard by electron release through the aromatic ring (see Setion 4.5.2). The amide analogue **55** (CMDA) was the first ADEPT prodrug evaluated clinically [139]. The later carbamate prodrug **56** (ZD 2767), releasing a more cytotoxic phenol iodomustard effector, showed growth delays of 14–28 days for LoVo colon carcinoma xenografts in nude mice, in conjunction with a CPG2/carcinoembryonic antigen antibody conjugate [140], and is currently in Phase I clinical trial [141].



6.2.3. Prodrugs for β-lactamase enzymes

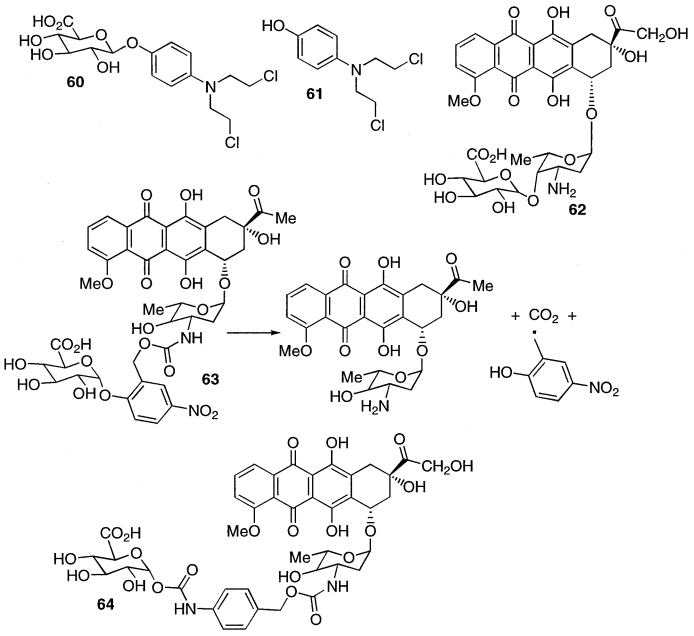
The ability of β-lactamase enzymes from *Enterobacter* species to selectively hydrolyse the four-membered β-lactam ring of penicillins and cephalosporins, resulting in spontaneous fragmentation of a carbamate side chain and release of amine-based effectors (*figure 9*), has been used in a variety of prodrugs [23, 142]. The carboxy and (when used) sulphoxide groups on the cephem nucleus assist with cell exclusion. A cephem prodrug (**57**) of the mitotic inhibitor desacetylvinblastine hydrazide was shown to be an excellent substrate for β-lactamase/antibody conjugates [143], generating substantial growth delays in KS174T and T430 human colon tumour xenografts [144]. Several nitrogen mustard prodrugs for β-lactamase have been evaluated; the cephem analogue (**58**) effected cures in mice bearing xenografts of human melanoma 3677 cells, if given subsequent to treatment with 96.5/bL, a mAb/β-lactamase conjugate that binds to specific surface antigens on these cells [145]. A study of a cephem derivative of doxorubicin (**59**) together with a variety of antibody/β-lactamase conjugates showed higher intratumoural levels of doxorubicin after treatment with the conjugate than with doxorubicin alone [146]. Prodrugs for other β-lactamase enzymes such as penicillin amidases have also been explored [23]. However, while this approach has many apparent advantages, including widely-available activating enzymes, good substrate specificity, limited endogenous enzymic interference and a well-defined mechanism of action, the differential cytotoxicities between drug and prodrug are generally only moderate, and this may be the reason why no such prodrugs have apparently proceeded to clinical trial.



6.2.4. Prodrugs for glucuronidase enzymes

Of a number of glycosidase enzymes, β-glucuronidase (GUS) has been the most widely used as an activating

enzyme in ADEPT. Because serum levels of the endogenous enzyme are very low (it is largely confined to lysozymes in cells), it is possible to use the human enzyme, thus avoiding potential immunogenicity problems [147]. A number of prodrug approaches have been explored [141]. The simple mustard prodrug (**60**) was 55-fold less toxic than the corresponding free drug **61** in human COLO 205 colon cancer cells [147], and could be fully activated by a mAb/*E. coli* GUS conjugate [148]. However, work has tended to focus on anthracycline effectors [149]. The epirubicin *O*-glucuronide prodrug (**62**) was 100–1000-fold less cytotoxic than epirubicin itself in vitro [150], but pretreatment of antigen-positive cells with an 323/A3-GUS-*E. coli* immunoconjugate again gave equivalent cytotoxicity to that of the free drug. The doxorubicin prodrug GLU-SP-DOX (**63**) used an immolative spacer unit [149], and although only 10-fold less cytotoxic than free doxorubicin, was a better substrate for the enzyme [151]. The doxorubicin prodrug DOX-GA3 (**64**) was 12-fold less toxic than doxorubicin in cells, in the human ovarian cancer cell line FMa, and was somewhat superior to doxorubicin itself against FMa xenografts in mice in conjunction with 323-A3/human β-glucuronidase conjugate [152]. However, the utility of such tightly DNA binding, cell cycle specific (topo II inhibitor) effectors is not yet known.



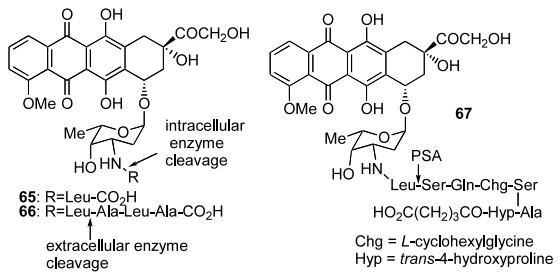
7. TAP activated by excreted tumour-specific enzymes

The role of the conjugated peptide in these prodrugs is not to physically locate the prodrug on tumour cells, but to serve as a substrate for designated enzymes that are produced and secreted preferentially by tumour cells. An early example was the prodrug **65** (Leu-Dox), where studies in A2780 human ovarian cancer xenografts in mice showed conversion to doxorubicin in tumour cells by cathepsin-like enzymes, resulting in higher AUC values (for free doxorubicin) and somewhat superior antitumour activity than with doxorubicin itself [153]. The prodrug gave lower levels of the cardiotoxic doxorubicin in heart tissues [154], and did receive a clinical trial [155].

In the related prodrug **66** (Super-Leu-Dox, SLD), the tetrapeptide NH_2-β-Ala-L-leu-L-Ala-L-leu ensures the hydrophilic prodrug is excluded from cells [156]. The tetrapeptide was developed empirically on the basis of rates of cleavage of various doxorubicin peptide conjugates by (undetermined) peptidases secreted from MCF7/6 human breast cancer cells. SLD is cleaved by these cells rapidly to the monopeptide **65**, previously shown (see above) to enter cells where it is cleaved to doxorubicin. In cell culture, prodrug **66** results in enhanced levels of doxorubicin in cells, and is superior to both **65** and doxorubicin

itself in its selective toxicity (IC_{50}) for MCF7/6 cells compared with fibroblasts. It was also superior to both drugs against MCF7/6 xenografts in vivo [156].

In the heptapeptide prodrug **67** (L-377,202), the peptide side chain was engineered to be a good substrate for the serine protease PSA (prostate-specific antigen), which is over-produced and excreted from prostate cancer cells. Cleavage by PSA of **67** at the Gln-Ser bond is expected to release NH_2-Ser-Leu-doxorubicin, but Leu-dox (**65**) was the main metabolite, suggesting rapid metabolism of the initially-formed product [157]. Prodrug **67** was much more cytotoxic towards the PSA-secreting LNCaP prostate cancer cell line than it was to non-PSA-secreting lines [158]. In LNCaP xenografts in vivo, the MTD of **67** was 7–10-fold higher than that of doxorubicin, and it was superior to doxorubicin in terms of lowering PSA levels and in reduction of tumour volumes [158]. In a Phase I trial of **67** in patients with advanced hormone-resistant prostate cancer, responses were seen in terms of reductions in PSA levels. Phase II trials are in progress, at a recommended dose of 225 mg/m^2 [159].



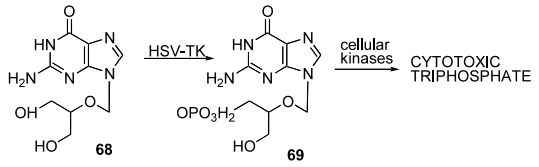


Figure 10. Activation of glanciclovir by HSV-TK.

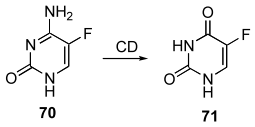


Figure 11. Activation of 5-flourouracil by cytosine deaminase.

8. TAP activated by exogenous enzymes delivered by gene vectors

8.1. Introduction

Of the very large number of "gene therapy" trials now in progress, a small but growing proportion are for gene-directed enzyme-prodrug therapy, or "suicide gene therapy" [160]. Much of the work reported has focused on development of the gene delivery systems, but this review will be restricted largely to the prodrugs. As noted above (Section 2.7), GDEPT imposes further demands on the design of prodrugs, which must be able to enter cells freely, yet not be substrates for the myriad of endogenous enzymes available.

8.1.1. Prodrugs for kinase enzymes

The non-toxic antifungal drug ganciclovir (**68**; *figure 10*) has been widely used as a prodrug in GDEPT protocols, in conjunction with the thymidine kinase enzyme from the Herpes simplex virus. This selectively converts it to the monophosphate (**69**), which can be converted by cellular enzymes to the toxic triphosphate (*figure 10*). This was one of the first GDEPT protocols developed, and has been tested in numerous clinical trials, primarily in gliomas by intratumoural injection [161]. A limitation of the approach is the poor bystander properties of the effector **69**, which cannot enter cells by passive diffusion, but uses gap junction connections [162] that are not well-developed in many tumour types [163].

8.1.2. Prodrugs for cytosine deaminase

This is another widely-studied GDEPT system, where the yeast enzyme cytosine deaminase [164] is used to selectively convert the non-toxic 5-fluorocytosine (**70**) to the thymidylate synthetase inhibitor 5-fluorouracil (**71**) (*figure 11*). This freely-diffusible effector confers greater bystander effects on the combination [165]. Because 5-fluorouracil is one of the most effective drugs for colon cancer, GDEPT studies focused mainly on the use of this combination in experimental colon cancer models. Studies with **70** in conjunction with a gene construct using cytosine deaminase under the control of carcinoembryonic antigen promotor/regulatory elements was active in xenografts of WiDr human colon carcinomas [166]. Possible drawbacks include the relatively low potency of the effector **71**, coupled with its pronounced cell cycle selectivity [167].

8.1.3. Prodrugs for oxidative enzymes

This work has focused largely on the cytochrome P450 enzymes, which are known to activate the clinical agent

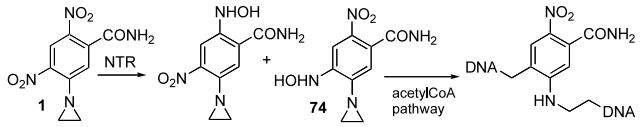
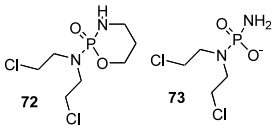


Figure 12. Metabolism of CB 1954 by E. coli NTR.

cyclophosphamide (**72**) [168]. This activation is normally non-specific, primarily in the liver, to generate the poorly-diffusing effector **73**. Treatment of sc 9L gliosarcoma tumours transduced with various isozymes (especially CYP2B6 or CYP2C18-Met) with **72** resulted in a large enhancement of the liver P450-dependent antitumour effect seen with control 9L tumours (growth delays of 25–50 days compared with 5–6 days), with no apparent increase in host toxicity [169].



8.1.4. Prodrugs for reductase enzymes

The aziridine **1** is activated by DT diaphorase (see Section 2.1) and to some extent by endogenous enzymes under hypoxia (see Section 4.5.2). However, it is activated much more efficiently [170] by the aerobic NTR from *E. coli* [171], in conjunction with NADH or NADPH, to a mixture of hydroxylamines (*figure 12*). The 4-hydroxylamine (**74**), is then further metabolised by cellular enzymes to DNA crosslinking species [7]. A large amount of work [172] with CB 1954 has established its high selectivity (up to 1000-fold) for a variety of cell lines transduced with the enzyme over the corresponding wild-type cell lines. It is now in clinical trial in conjunction with NTR, using a GDEPT protocol [173]. The related mustard **35** (SN 23862) is an even better substrate for NTR [174], and shows high selectivity for NTR-positive cells [175] and a significant bystander effect in cell culture [176].

The *E. coli* NTR also metabolises a variety of 4-nitrobenzyl carbamate derivatives (see Section 4.5.3), despite the low reduction potentials (ca. −490 mV) [177], to release amines [178]. A variety of these have been proposed as GDEPT prodrugs in conjunction with NTR, to release effectors such as actinomycin and mitomycin [179], enediynes [180], amino-seco-cyclopropylindolines [181] and tallimustines [182]. The nitroimidazole RSU-1069 (**24**; racemate) has also been studied as a GDEPT prodrug in conjunction with human P450 reductase, which activates it under hypoxic conditions [183].

9. Conclusions

The above article has reviewed primarily the reported strategies for TAP, concentrating mainly on the prodrug chemistry. The oldest strategy is probably the immunotoxin approach (Section 6.1). As noted above, this required improvements in a number of the enabling technologies before becoming useful, and is now engendering much clinical interest through conjugates such as **47**–**49**. The exploitation of tumour-specific secreted proteases to activate drug/peptide conjugates uses somewhat similar technology, but is conceptually different (Section 7), and is too new for its full potential to be evaluated. The ADEPT concept (Section 6.2) is a more direct descendant of the immunotoxin approach, and has also proved difficult to master, but now also has promising drugs such as **56** in clinical trial. GDEPT (Section 8) is another variant with a promising future, but gene delivery issues (outside the scope of this review) still have to be improved. Finally, work over many years on TAP activated by tumour hypoxia (Section 4) is beginning to be rewarded by the progression of the bioreductive agent tirapazamine (**3**) to Phase III trials. The related approach of TAP activation by radiative bioreduction (Section 5) remains to be proven. Overall, TAP are beginning to show promise for cancer therapy.

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