Aerobic Nitroreduction by Flavoproteins: Enzyme Structure, Mechanisms and Role in Cancer Chemotherapy

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Abstract: NQO1 (DT-diaphorase) and its truncated isoenzyme, the metalloenzyme NQO2, can reduce quinone substrates by two-electron transfer. While NQO1 is a known detoxification enzyme, the function of NQO2 is less well understood. Both rat NQO1 and human NQO2 reductively bioactivate the dinitroarene CB 1954 to a cytotoxic product that behaves as a difunctional DNA-crosslinking species with potent anti-tumour activity, although human NQO1 is much less effective. A FMN-dependent nitroreductase from *E. coli B* also reduces quinones and reductively bioactivates CB 1954. However, this enzyme reduces CB 1954 to the 2- and 4-hydroxylamines in equivalent yield, whereas NQO1 and NQO2 generate only the 4-isomer.

The reduction profile is a key factor in the development of anti-tumour prodrugs, where distinct delivery strategies are being evaluated: prodrug therapy, antibody-, macromolecule- and gene-directed enzyme prodrug therapy (ADEPT, MDEPT or GDEPT). The flavoprotein enzymes are explored in terms of structure and bioreduction mechanism, particularly for use in the design of novel prodrugs with potential application as chemotherapeutic agents.

INTRODUCTION

The flavoenzyme NQO1 [NAD(P)H:quinone acceptor oxidoreductase, or DT-diaphorase (EC 1.6.99.2)] is important in the bioactivation and detoxification of anti-cancer prodrugs and certain xenobiotics [1]. The ubiquitous FAD-containing enzyme catalyses obligatory NAD(P)H-dependent two-electron reductions of quinones to hydroquinones, bypassing the toxic semiquinone radical species generated during one-electron reductions catalysed by cytochrome P450 and xanthine oxidase enzymes. By this mechanism, NQO1 protects cells against the damaging effects of reactive oxygen species and free-radicals [2]. Levels of the enzyme's activity are raised significantly when cells are challenged by a variety of potentially carcinogenic compounds, including isothiocyanates, which are present in cruciferous vegetables such as broccoli [3,4]. High NQO1 activity has been reported in human tumour cell lines of breast [5–7], brain [8], colon [5,6,9,10], lung [5–7] and liver origin [5,6,11,12]. There is a marked increase in the activity of NQO1 in human colonic carcinomas when compared with the enzymatic activity of the surrounding normal colonic mucosa [13]. Further, NQO1 levels in bone marrow are low [1,14], directing toxicity away from tissues that are usually sensitive to conventional cytotoxic chemotherapy. Because NQO1 is constitutively over-expressed it is currently being exploited as a target in the development of anti-cancer prodrugs. Cells expressing high levels of NQO1 are also sensitive to drug treatment; thus, the cytotoxic anti-tumour quinones, the mitomycins, aziridinylbenzoquinones and anthracyclines are bioactivated by NQO1 [1]. However, these agents can also be activated by other enzyme systems such that the tumour selectivity conferred by the raised expression of NQO1 can here be lost or masked [15–17].

One bioreducible compound that appears to be *selectively* activated by NQO1 is CB 1954 [5-(1-aziridinyl)-2,4-dinitrobenzamide, Fig. **1**)]. In this case, the compound can be activated in air by reduction of the 4-nitro group, to generate 5-(1-aziridinyl)-4-hydroxylamino-2-nitrobenzamide [18]. The effect of such aerobic nitroreduction is to convert a chemically weak monofunctional-alkylating agent (aziridine moiety) to a potently reactive difunctional alkylating species. An exquisite 10^4-fold increase in drug cytotoxicity has been demonstrated as a direct consequence of this bioactivation process [19].

ENZYMATIC BIOACTIVATION OF THE PRODRUG CB 1954

The dinitrobenzamide CB 1954 represents one of the very few examples of an agent to effect a genuine and demonstrable anti-tumour selectivity, where this constitutes a vital requirement for any chemotherapeutic anti-cancer agent [20]. Whilst structurally only a monofunctional alkylating agent towards nucleophiles (by virtue of its single aziridine ring substituent), CB 1954 showed a dramatic and highly selective activity against the rat Walker 256 tumour and could actually cure this tumour. Such selectivity was unprecedented for any monofunctional- alkylating agent, and it was evident that the sensitivity of the Walker tumour towards CB 1954 pointed to a unique biochemical feature.

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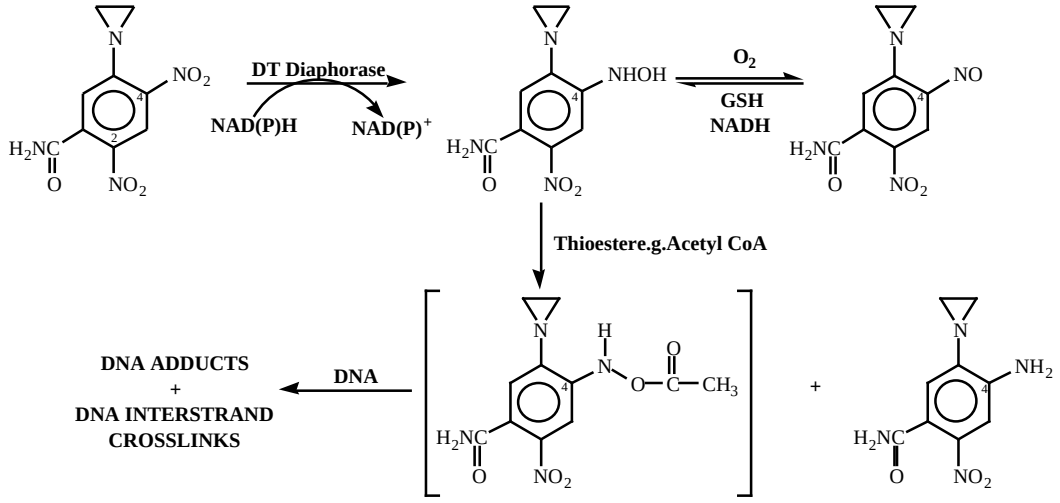

Fig. (1). The bioactivation of CB 1954. The initial step is the nitroreduction by the enzyme DT-diaphorase (NQO1) to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This hydroxylamine can react with thioesters to produce a DNA-reactive species. It is postulated that this is the *N*-acetoxy derivative [29].

The prospect that a human tumour could be found that shared the sensitivity of the Walker tumour has made the mechanism of action of action of CB 1954 the subject of continual interest for more than 20 years. Indeed, CB 1954 has been described as "a drug in search of a human tumour to treat" [21].

CB 1954 arose from a large series of *N*-substituted aziridine derivatives synthesised in the early 1950's and investigated for their tumour growth-inhibitory activity against the Walker 256 carcinoma *in vivo* at the Chester Beatty Laboratories in London. Although the most pronounced anti-tumour effects were observed for compounds containing at least two aziridine residues, an exception to this requirement for difunctionality was afforded by the monofunctionalised agent 1-(1-aziridinyl)-2,4-dinitrobenzamide (CB 1837), which showed significant anti-tumour activity [22]. CB 1837 was found to have a therapeutic index (*TI* = 10) that was comparable to many agents in current clinical use [e.g., chlorambucil (*TI* = 7), cyclophosphamide (*TI* = 22), melphalan (*TI* = 9) and cisplatin (*TI* = 14)].

During evaluation of a large series of structurally related compounds, a derivative carrying a carboxamide substituent (CB 1954, (Fig. **1**) was shown to have an even higher therapeutic index against the Walker 256 carcinoma (*TI* = 70). Such activity was greater than found for any known compound [23,24]. Thus, CB 1954 was discovered in a drug design exercise attempting to improve the water solubility of CB 1837 by the introduction of a polar carboxamide residue.

However, although CB 1954 was shown to have high potency and specificity of action against Walker tumour cells both *in vivo* and *in vitro*, it was established at an early stage that CB 1954 was ineffective against a range of other animal and human tumours – even those that respond to the growth-inhibitory effects of difunctional alkylating agents [25]. In addition to its potent and specific activity, this agent exhibited minimal toxic effects on the haematopoietic system. Pathological effects, at toxic doses in rodents, were observed in the liver and urinary tract epithelium; this behaviour contrasts with the intestinal epithelium toxicity observed with many alkylating nitrogen mustards. CB 1954 was found to be 10-fold more toxic in the rat than the mouse. This was reflected in the higher TI value for this compound against the Walker tumour when grown and treated in mice (reviewed by [25]).

The specificity of CB 1954 against Walker cells has also been demonstrated in tissue culture, thereby eliminating any role the host may play in activating the drug. *In vitro*, Walker cells are ~10^5-fold more sensitive towards CB 1954 than are, for example, Chinese hamster V79 cells. It was in this cell system that the actual reason why Walker cells were sensitive to this drug was first shown, when it was demonstrated that CB 1954 formed DNA–DNA interstrand crosslinks in Walker cells but not in the insensitive V79 cells [26]. Thus, in Walker cells, CB 1954 is converted from a monofunctional agent to a difunctional DNA-reactive agent. Co-culturing Chinese hamster V79 cells with Walker cells in the presence of CB 1954 resulted in the sensitisation of the V79 cells towards CB 1954. Crosslinks were now present in their DNA [18], indicative of the formation in Walker cells of a diffusible toxic metabolite of CB 1954. It was suggested that this activation was occurring by reduction of the nitro groups of CB 1954 to provide an additional reactive centre [26].

Confirmation of a selective bioactivation process was demonstrated by (i) purification of the nitroreductase enzyme responsible for this action from Walker cells, and (ii) identification of the activated CB 1954 form. The enzyme was identified as NQO1 [27] where, in the presence of either NADH or NADPH, it catalyses the aerobic reduction of CB 1954 to its 4-hydroxylamino derivative (Fig. **1**) [18]. 5-(1-

Aziridinyl)-4-hydroxylamino-2-nitrobenzamide is highly cytotoxic, even to those cells resistant to CB 1954, and can induce interstrand crosslinks in their cellular DNA. Thus, it is the formation of this enzymatic nitroreduction product that accounts for the atypical sensitivity of Walker cells towards CB 1954. Irrespective of their inherent ability to bioactivate CB 1954, all cell types so far examined have a comparable sensitivity towards the reduced 4-hydroxylamino derivative [28].

While 5-(1-aziridinyl)-4-hydroxylamino-2-nitrobenzamide (Fig. **1**) can induce formation of DNA–DNA interstrand crosslinks in cells, it does not effect such lesions in naked DNA [29]. This suggested that there is a further activation step in cells, this converts the nitroreduction product to the active proximal DNA-crosslinking, cytotoxic species. This notion was supported by the absence of a linear dose response in crosslinking in cells treated with this agent, consistent with the saturation of a secondary activation step. An enzymatic esterification and activation of the hydroxylamine was proposed, by analogy with events implicated in the metabolic conversion of 4-nitroquinoline-*N*-oxide or *N*-acetylaminofluorene [29]. In fact, 5-(1-aziridinyl)-4-hydroxylamino-2-nitrobenzamide can be activated non-enzymatically, to a species that can produce interstrand crosslinks by reaction with naked DNA, by a direct chemical reaction with acetyl-coenzyme A and other thioesters [29]. The ultimate, DNA-reactive species generated from CB 1954 is thus probably 4-(*N*-acetoxy)-5-(1-aziridinyl)-2-nitrobenzamide (Fig. **1**)). A further product of this reaction with thioesters is the 4-amino derivative, as the result of a competitive activation process (Fig. **1**). Interestingly, 4-amino-5-(1-aziridinyl)-2-nitrobenzamide is the major urinary metabolite of CB 1954 in the rat [30].

The bioactivation of CB 1954 results in a vast increase in drug cytotoxicity, where the resulting dose modification can be up to 10^4-fold. This effect is greater than would be predicted even by conversion of a mono- to a difunctional agent. Where monofunctional congeners of difunctional agents are available, as with 'half'-mustards and monofunctional platinum compounds, the dose modification for equitoxicity is seen to be only ~50–200-fold [31,32]. Experimental findings regarding the properties and formation of interstrand DNA crosslinks explain why cells that are able to bioactivate CB 1954 are so cytotoxically affected.

(1) The CB 1954-induced interstrand crosslink is formed with a very high frequency, and can contribute up to 70% of the total lesions [33]. This frequency is much higher than reported for other agents (e.g., interstrand crosslinks represent <2% of the total DNA reactions of cisplatin or carboplatin [34]). The interstrand crosslink is, in terms of molar efficacy, a more intrinsically toxic lesion than either single-strand diadducts and monofunctional lesions. Any agent that induces a very high proportion of crosslinks would thus be expected to be more toxic than a compound that effects only a low frequency.

(2) The crosslinks are poorly repaired, which may be cause them to be even more intrinsically cytotoxic than those induced by other difunctional agents [33]. In direct support of this finding, induction of interstrand DNA crosslinks has recently been shown for a series of potent dimers generated from pyrrolo[2,1-*c*][1,4]benzodiazepine sub-units [35–41]. In this case, alkylation events tailored to be mediated in the DNA minor groove result in crosslink lesions that pose a similarly difficult hurdle for cellular repair. Notably, the cytotoxic potency and hence chemotherapeutic response is here also correlated with the yield of crosslinks (difunctional reactivity) rather than mono-alkylated DNA adducts [36,38,42–44].

(3) As a consequence of the bioactivation of the CB 1954, there is a 10-fold increase in the amount of DNA-bound drug in Walker cells, as compared to cells which cannot reduce CB 1954 [33].

The unusual profile for CB 1954-induced interstrand DNA crosslinks suggest that this lesion is unlike those formed by other agents. The interstrand crosslink lesion(s) induced by CB 1954 have yet to be fully identified. However, the 4-hydroxylamine (after activation as detailed) reacts predominantly with the C8 position of deoxyguanosine. In double-stranded DNA, this would leave the aziridine function poised to react with residues on the opposite strand to form the observed crosslinks. Extensive molecular modelling studies indicate that this second-arm reaction will preferentially be at the O6 position of a deoxyguanosine on the opposite DNA strand in the major groove conduit (Fig. **2**; T.C. Jenkins and R.J. Knox, unpublished data). Such a C8–O6 interstrand DNA crosslink is unique, not being produced by any other alkylating or platinating agent, and would account for the cytotoxic potency. These properties, coupled with the selectivity of the bioactivation step, explain why CB 1954 is so exceptionally effective as an anti-tumour agent in the rat and capable of curing the Walker carcinoma.

Elucidation of NQO1 as the bioactivating enzyme for CB 1954 renewed the possibility of identifying human tumour types with a sensitivity similar to the Walker carcinoma. This was because, as discussed above, this enzyme is known to have a favourable distribution in human tumours.

The rat and human forms of NQO1 have been cloned and sequenced; the human NQO1 cDNA and proteins are 83% and 85% homologous with the rat liver cytosolic cDNA and protein, respectively [45]. Both enzymes are inducible cytosolic flavoproteins encoded by a single gene. The human protein is biochemically very similar to the rat protein, with only small differences between K_m values determined for the substrates menadione and NADH [45]. Thus, it might be predicted that human or rat NQO1 would metabolise CB 1954 in a manner similar to the protein from Walker cells, and that the cytotoxicity resulting from the bioactivation of CB 1954 might be observed in human tumours expressing significant levels of this enzyme.

A number of human cell lines were shown to contain NQO1 at levels comparable to those found in Walker and certain other rat cell lines [28]. The rat cell lines were all sensitive to CB 1954 and the resulting cell killing approached that obtained in Walker cells; thus, Walker cells are not uniquely sensitive towards CB 1954. Conversely, all

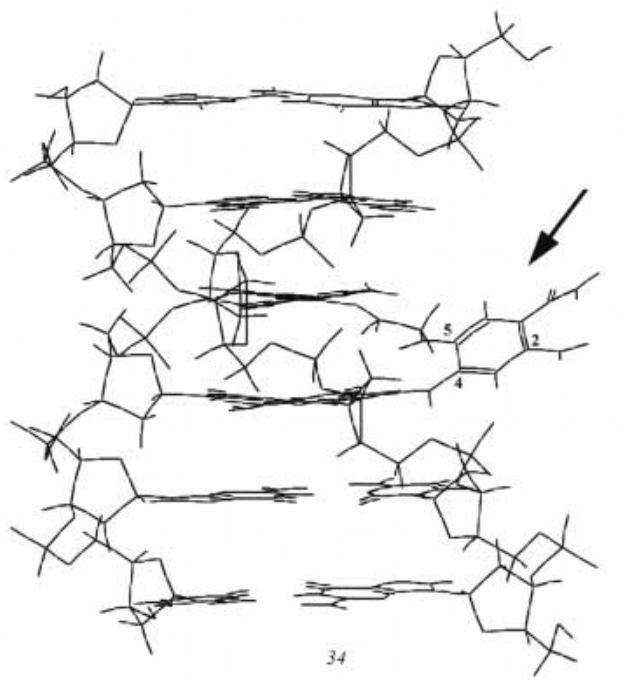

Fig. (2). Proposed structure for the interstrand DNA crosslink produced by CB 1954. After activation, the 4-hydroxylamine reacts predominantly with the C8-position of deoxyguanosine. Modelling indicates that the aziridine group can then react on the O6-position of a deoxyguanosine on the opposite DNA strand to produce the observed crosslinks. Such damage is unique, and could explain why (i) CB 1954 produces a very high crosslink frequency, and (ii) these crosslinks are very persistent in cells and not readily removed by DNA repair processes.

human cell lines were dramatically less toxically affected by CB 1954, with a 500–5000-fold higher dose of the agent being required to effect a cytotoxic response comparable to that in cells of rat origin [28]. In contrast to the large difference in their cytotoxic response towards CB 1954, both the rat and human cell lines were similarly affected by the 4-hydroxylamino derivative of CB 1954 [28]. The sensitivity of human cells toward 5-(1-aziridinyl)-4-hydroxylamino-2-nitrobenzamide suggested that their resistance towards CB 1954 was due to neither any failure to further activate the hydroxylamine nor to an intrinsic resistance to the formed DNA adducts. It was therefore suggested that CB 1954 is reduced differently by the human and rat forms of NQO1 [28].

To investigate this proposal, the human form of NQO1 was purified to homogeneity from Hep G2 cells [28]. Although NQO1 has been studied extensively, the most common source of the enzyme is rat liver and information regarding the human protein was limited. However, as might be predicted from the large degree of homology between the rat and human forms of NQO1, the biochemical properties of the Hep G2 and Walker forms of the enzyme, with respect to co-factors and the reduction of menadione, were very similar [28]. In contrast, significant differences were observed in the ability of NQO1 isolated from either Hep G2 or Walker cells in their ability to reduce CB 1954 to the active 4-hydroxylamine product. Although, both enzyme forms generated the 4-hydroxylamino derivative as the single product, the human Hep G2 form of the enzyme was intrinsically less able to carry out this reduction and the k_{cat} value is >6-fold higher for the Walker cell form of the enzyme (0.068 sec^{-1}) than for the human NQO1 (0.0107sec^{-1}) (Table **1**). The inability of the human enzyme to generate the required cytotoxic species from CB 1954 accounts for the intrinsic lack of sensitivity of human cells towards this agent.

Table 1. Kinetic parameters for NQO2, *E. coli* nitroreductase, human and rat NQO1 with respect to CB 1954. NRH was used as co-substrate for NQO_2. Values for the other enzymes were determined using NADH

Enzyme	K_m (μM)	k_{cat} (s^{-1})
NQO2	263±13	6.01
Nitroreductase	862±145	6.0
Rat NQ01	826	0.0683
Human NQ01	1403	0.0107

By using *Escherichia coli*-expressed (recombinant) forms of NQO1 and evaluating them under identical conditions, it was confirmed that the human enzyme was not as effective as the rat enzyme in reducing CB 1954, although the two enzymes have similar NAD(P)H-menadione reductase activities [45]. Interestingly, although the amino acid sequence of mouse quinone reductase is more homologous to that of the rat enzyme, it was found that the mouse enzyme behaves similarly to the human enzyme in its ability to reduce these compounds and to cause drug-induced DNA damage [45]. In order to identify the region of quinone reductase responsible for the catalytic differences, two mouse–rat chimeric enzymes were generated: (i) MR-P, with mouse amino-terminal and rat carboxy-terminal segments of quinone reductase, and (ii) RM-P, with rat amino-terminal and mouse carboxyl-terminal segments of quinone reductase. The chimeric enzymes were shown to have catalytic properties resembling those of rat and mouse quinone reductase, respectively. Thus, the carboxyl-terminal portion of the enzyme plays a key role in the reduction of cytotoxic drugs and the binding of flavones [45]. Using site-directed mutagenesis to replace residues in the rat enzyme with the human sequences and residues in the human enzyme with the rat sequences, it was shown that residue 104 (tyrosine in the rat enzyme, and glutamine in the human and mouse enzymes) is a very important residue responsible for the catalytic differences between the rat and the human (and mouse) enzymes. With an exchange of a single amino acid, the rat mutant Y104Q behaved like the wild-type human

enzyme, and the human mutant Q104Y behaved like the wild-type rat enzyme in their ability to reductively activate CB 1954 [46].

To investigate if the resistance of human tumour cells to CB 1954 could be fully accounted for by the properties of human NQO1, a cell-line panel was made consisting of V79 cells engineered to express either the human or rat enzyme forms. V79 cells have practically no measurable NQO1 activity [14] and thus provide a suitable null background. A control cell line was also constructed with the empty expression vector. Thus, the panel consisted of cell-lines expressing various levels of either rat or human NQO1 in an identical cellular background [47] (Fig. **3**). The sensitivity of these various cell lines towards CB 1954 was determined. On the basis of these IC_{50} values, the cytotoxic effect of CB 1954 is proportional to the activity of either the rat or human enzyme (Fig. **3**) [47]. Cells expressing the rat enzyme were more sensitive than cells expressing the human enzyme at a comparable level of menadione oxidoreductase (NMOR) activity. At the high levels of NMOR activity reported in tumour cell lines (about 20,000 U/mg cytosolic protein [28]) there is a 10^4-fold difference in the concentration of CB 1954 required to produce the same cytotoxic response in cells expressing the rat as opposed to the human form of NQO1 (Fig. **3**) [47]. These findings show that the resistance of human tumours to CB 1954 can be accounted for solely by the kinetic properties of the enzyme for this prodrug, and that there is no need to invoke other mechanisms of resistance [47]. Thus, a *single* amino acid change accounts for the inherent lack of sensitivity of human tumours towards CB 1954!

QUINONE REDUCTASE TYPE 1 (NQO1) – STRUCTURE AND MECHANISM

In order to understand the reasons for these catalytic differences at the molecular level the three-dimensional X-ray crystal structure of the human enzyme [48,49] was compared with that of rat NQO1 complexed with (a) the triazine dye cibacron blue, a competitive inhibitor with respect to NAD(P)H and duroquinone, and (b) $NADP^+$ [50]. There are 37 single amino acid changes between the human and rat forms of NQO1. In both species the enzyme is a tightly-associated physiological dimer, with each monomer containing a non-covalently bound flavin adenine dinucleotide (FAD) prosthetic group essential for catalytic activity. While the FAD is somewhat more buried in the active site for the human enzyme compared to the rat enzyme, the two conformations are identical.

The enzyme name of NQO1 arose from of its (then) unique ability to use either NADH or NADPH (at the time abbreviated as DPNH and TPNH, respectively) as co-factors in the reduction of quinone substrates. As well as its ability to use either co-factor, this enzyme is also remarkable in that it can simultaneously transfer two electrons to its substrate. The primary cellular role of this enzyme appears to be the catalytic detoxification of quinones by two-electron

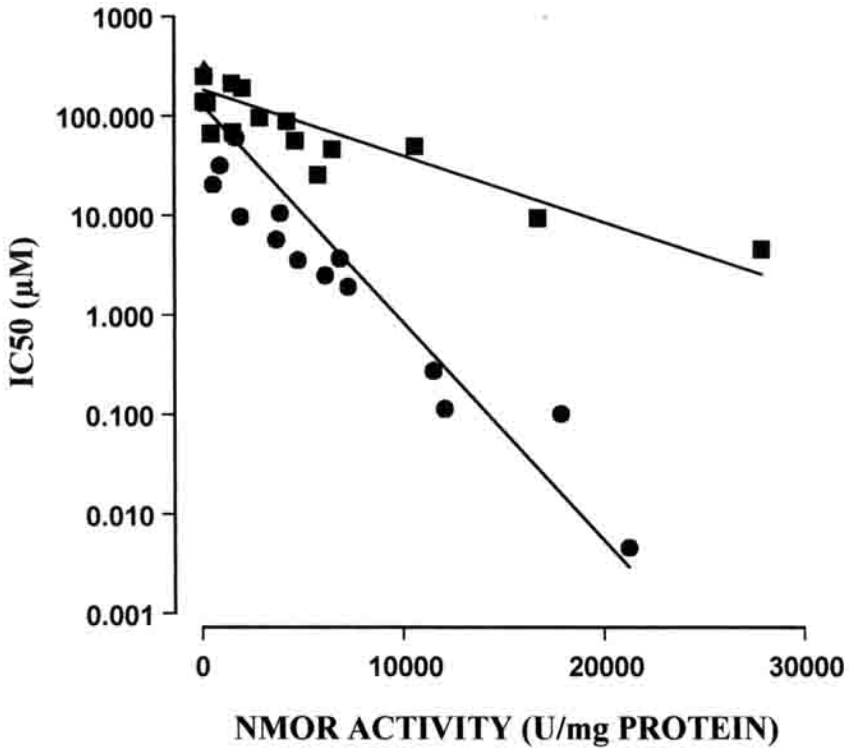

Fig. (3). The sensitivities of individual clones of transfected V79 cells expressing either rat or human NQO1 (DT-diaphorase) to CB 1954 [47]. Cells expressing rat NQO1 (l) or human NQO1 (■). NMOR activity is in units of nmol cytochrome C reduced per minute. IC_{50} values are for a 72-h exposure to CB 1954. Error bars have been removed for clarity.

reduction to their corresponding hydroquinones. This process avoids the cytotoxicity that would stem from the redox cycling produced by a one-electron reduction of quinones through formation of superoxide radicals by transfer to O_2. Presumably, the mechanism by which NQO1 can directly transfer two electrons to a quinone substrate is also implicated in the 4-electron reduction of the dinitroarene CB 1954. However, reduction of CB 1954 is intrinsically $>10^4$-fold slower than for the reference menadione compound (a quinone), and transfer of reducing equivalents from the enzyme to CB 1954 is slow and very rate-limiting [51]. This relatively slow rate of reduction may be required to avoid saturation of the second activation step discussed above.

Analysis of the protein structure determined for rat NQO1 revealed that the component monomers consist of two separate domains: a major catalytic domain (residues 1–220) and a small, C-terminal domain (residues 221–273). This finding confirmed an earlier model proposed on the basis of proteolytic digestion studies [52]. The overall fold of the catalytic domain is similar to that for other flavoproteins, consisting of a central, twisted parallel ß-pleated sheet surrounded by helices. Interestingly, the exact topology resembles that of the bacterial FMN-dependent *Clostridium* flavodoxin [50], but not that of other FAD-bound proteins whose structures have been determined.

The active site is situated at the interface between the dimers in both the human and rat enzymes and involves residues from the polypeptide chains of both monomers. The carbonyl group of the nicotinamide of $NADP^+$ (O7N) is involved in hydrogen bonding with both the OH of Tyr126 and with the OH of Tyr128 of the second monomer. Further, the side-chain of Phe178 of the second monomer stacks against the nicotinamide ring. Residues forming the C-terminal domain provide the binding site for the hydrophilic portions of NAD(P)H: the adenine of the AMP moiety interacts with residues in the main chain of the loop connecting strands 8 and 9, and the ribose makes contacts with Phe232 and Phe236.

The X-ray structures of the two rat complexes provide an explanation for the obligatory two-electron reduction of substrates via a 'ping-pong' mechanism. It is proposed that in the first half of the reaction cycle there is a direct hydride transfer from NAD(P)H to FAD; in the second half, there is subsequent hydride transfer from $FADH_2$ to the quinone substrate, which occupies the site vacated by the oxidised NAD(P)H. However, this mechanism is consistent with that of flavodoxin rather than flavoenzymes, which usually bind both NAD(P)H and their respective substrates simultaneously. Furthermore, the positions of the flavin moieties are closely similar in diaphorase and flavodoxin.

Comparison of the active sites of the human and rat NQO1s revealed some differences in volume and shape which would be a likely factor in determining the optimal size for a substrate (or drug). These changes are a direct result of substitutions in the hydrophobic side-chains V69A and L120F that form the active-site boundary. Residues Trp105, Phe106 are conserved in both the rat and human enzymes, and together with the main chain carbonyl of Leu103 (also conserved in both species) interact directly with the flavin rings to stabilise the isoalloxazine moiety. Gln104, which contributes to the stabilising of the isoalloxazine in human diaphorase, is a tyrosine in the rat enzyme, and it is this residue that is implicated by site-directed mutagenesis to be responsible for the catalytic differences between the two species. An H-bond formed by a water molecule situated between Tyr104 and a phosphate oxygen (O3') of FAD is consequently absent from the human structure, resulting in a ~0.7 Å shift in the FAD position between the two structures. Molecular models of a CB 1954 molecule positioned in the active site of the human structure suggested that this minor shift in flavin position alters the distance between the drug and the His161 residue, a histidine implicated in the transfer of a proton in the two-electron reduction mechanism [48,50].

QUINONE REDUCTASE TYPE 2 (NQO2) – A LATENT NITROREDUCTASE ENZYME

A further CB 1954-reducing enzyme is present in human tumour cells [53], with much greater activity than attributable to NQO1; however, the enzyme is latent and only detectable in the presence of dihydronicotinamide riboside (NRH). The enzyme responsible for this activity is human NAD(P)H quinone oxidoreductase 2 (NQO2) [53], which was identified by its homology to NQO1 [54]. The last exon in the NQO2 gene is 1603 bp shorter than the last exon of the NQO1 gene and encodes for 58 amino acids as compared to 101 amino acids encoded by the NQO1 gene; the NQO2 protein is thus 43 amino acids shorter than the NQO1 protein. The high degree of conservation between NQO2 and NQO1 gene organisation and sequence confirmed that the NQO2 gene coded for a second member of the NQO gene family in humans. However, it lacks the quinone reductase activity characteristic of NQO1, and appeared to have little enzymatic activity [55]. The apparent lack of activity is because NQO2 uses NRH (nicotinamide riboside (reduced); Fig. **4**) and *not* NAD(P)H as an electron

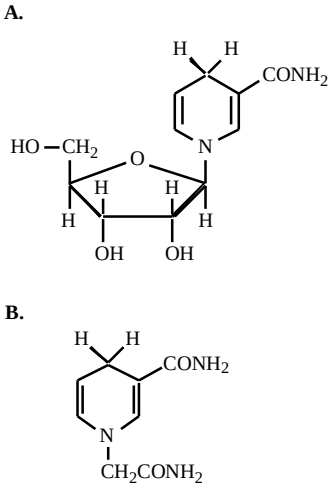

Fig. (4). (A) Dihydronicotinamide riboside (NRH), and (B) EP-0152(R). These compounds act with human NQO2 to reduce CB 1954. In contrast, the biogenic co-substrates NADH and NAD(P)H are not effective with this enzyme.

donor. This is a novel and unique property as NRH is a non-biogenic compound. Interestingly, an NRH-metabolising activity described in bovine kidney in the early 1960s [56,57] has now been ascribed to NQO2 [58]. In the presence of NRH, NQO2 can catalyse a two-electron reduction of quinones and the four-electron nitroreduction of CB 1954 [53]. NQO2 is 3000-fold more effective than human NQO1 in the reduction of CB 1954 under standard assay conditions (100 μM CB 1954, 500 μM co-substrate) [53]. In this respect, NQO2 resembles the *E. coli* nitroreductase but, like NQO1, it forms only the 4-hydroxylamine derivative. Further, NQO2 has a higher apparent affinity for CB 1954 than either NQO1 or *E. coli* nitroreductase (Table **1**). In the absence of NRH, NQO2-expressing human tumour cells remain insensitive towards CB 1954. Addition of NRH (itself non-cytotoxic) can increase the cytotoxicity of CB 1954 by $>10^4$-fold (Fig **5**) [59].

In addition to their different co-factors (electron donors), there are also differences in substrate and inhibitor specificities [57]. NQO2 has no affinity for dicoumarol, a competitive inhibitor of NQO1 with respect to NAD(P)H, and cibacron blue, a powerful inhibitor dye for NQO1 only weakly inhibits NQO2. Interestingly, the crystal structure of NQO2 was found to contain a hitherto undiscovered metal-binding site, not found in NQO1 [60]. The metal site has a distorted tetracoordinate geometry, similar to type-1 copper sites found in blue copper proteins such as plastocyanin and ceruloplasmin, involving two histidine ligands, one cysteine, and a main-chain carbonyl oxygen. It is therefore suggested that NQO2 is a copper enzyme with the metal-ion, which is located at the surface of the protein, linked to the active site and participating in the transfer of electrons. However, the purified-recombinant enzyme contains zinc rather than copper, although this may be due to the incorporation of non-native metal during heterologous expression, as is known to occur in the copper-binding protein, azurin [61].

In many other respects NQO2 resembles NQO1 in its overall structure. The overall topology of the major catalytic domain is essentially similar, but NQO2 entirely lacks the C-terminal domain of NQO1 and the 10 terminal residues, which are involved in metal co-ordination, share no sequence homology. The binding of FAD is similar in both enzymes, with the isoalloxazine hydrogen-bonded to main-chain NH groups of Trp105, Phe106, Gly149 and Gly150. As in NQO1, the carbonyl oxygen of Leu103 contacts N5 of the isoalloxazine ring; furthermore, Tyr104 of the rat NQO1, mutated to a glutamine in the human enzyme, is conserved in human NQO2, with its side-chain being stacked with the dimethylated ring of the flavin. As expected, residues involved in the binding of NAD(P)H in NQO1 are not well-conserved in NQO2 due to loss of the C-terminal contacts, specifically those involving the ADP moiety.

A truncated form of NQO1 was constructed and found to be able to use NADH as electron donor, but at a slower rate compared with the full-length enzyme [62]. Such truncation may explain the weak inhibition determined for NQO2 with the competitive inhibitors dicoumarol and cibacron blue, and indicates that the C-terminal domain of NQO1 is both important for enzyme catalysis and more important for NADH oxidation than NRH oxidation. Thus an additional region(s) is also involved in differentiating the binding of NADH *versus* NRH to NQO1 and NQO2 [62]. Non-phosphorylated hydride donors are believed to bind NQO2 through aromatic-stacking interactions although the mechanism for generating the reduced forms is uncertain. There are a number of differences in the active sites of NQO1 and NQO2: Tyr126 and Tyr128, whose side-chains are perpendicular to the plane of the isoalloxazine ring in NQO1, are replaced by Phe126 and Ile128 in NQO2, thereby providing a larger and more hydrophobic cavity.

Although NRH is highly water-soluble and shows little (if any) acute toxicity it is expensive and time-consuming to synthesise. As a result, other reduced pyridinium compounds have been investigated as replacements and assessed with regard to their (i) ability to act as a co-substrates for NQO2; (ii) chemically stability; (iii) inherent toxicity; (iv) pharmacological profile, and (v) ease of synthesis. Simple dihydropyridine derivatives have now been shown to similarly behave as co-substrates for bioreduction by NQO2. In its reduced form, the simplest quaternary salt (and therefore reducible) derivative of nicotinamide, 1-methyl-3-carbamoylpyridinium, was an efficient co-substrate for NQO2 with a specific activity about 30% that of NRH [59]. Alteration in chain length and/or steric bulk at the 1-position of the nicotinamide ring resulted in improved specific activity such that compounds more active than NRH were identified [59]. However, there was a limit to this effect with little activity found with either the analogous 1-phenyl or 1-benzyl derivatives [59]. These findings suggest that the co-substrate binding site of NQO2 is sterically constrained, and would explain why neither NADH nor NADPH are effective co-substrates for this enzyme. Little enzyme activity is seen with any nicotinic acid derivative, suggesting that a negative charge at the 3-position of the pyridine ring is also poorly tolerated within the enzyme binding site [59].

Most of the dihydropyridines examined were less chemically stable than NRH and oxidised in aqueous solution to restore their parent pyridinium compounds [59]. However, the 1-carbamoylmethyl derivative (designated EP-0152R, (Fig. **4**) was more stable than NRH [59]. Because of its specific activity EP-0152R was selected for further evaluation [59]. In cytotoxicity assays EP-0152R was non-cytotoxic like NRH and in animal studies no acute toxicity was observed at doses up to 800 mg/kg [59,63]. Therapeutic treatment of PC-3 human tumour xenograft was also achieved using a daily (×4) dosing schedule of 25 mg/kg CB 1954, followed by 250 mg/kg of either NRH or EP-0152R. In the presence of either co-substrate, CB 1954 showed a substantial growth delay, with EP-0152R giving a superior therapy than NRH (Fig. **6**) [63].

The serum half-life of NRH in the mouse is only ~7.5 min, although this is improved to ~10 min with EP-1052R [63]. In the experiments described above, the half-lives of these compounds are probably too short for i.v. administration to be fully effective. It should also be noted that the results are for a single cycle of treatment. However, the combination of CB 1954 and an otherwise inert co-substrate for NQO2 appears to be highly effective despite such experimental limitations.

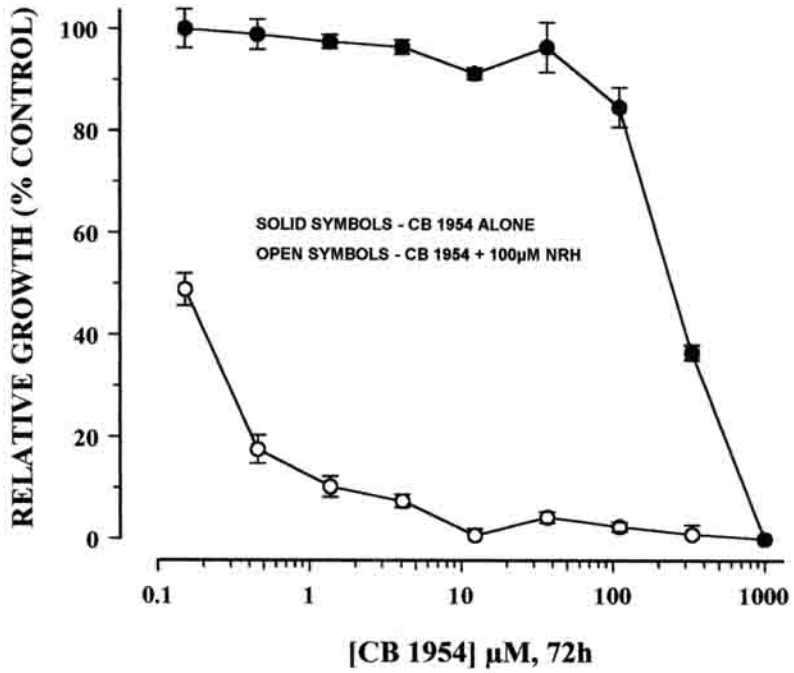

Fig. (5). The sensitivity of an NQO2-expressing cell line to CB 1954 in the absence and presence of NRH. Cells were exposed to the agent for 72 h at 37°C [59].

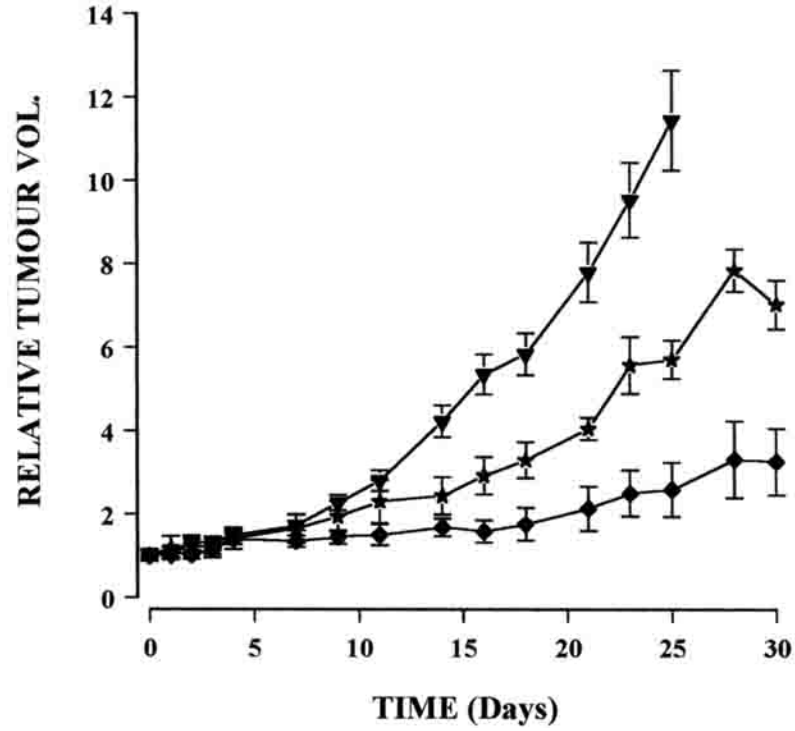

Fig. (6). Therapy of PC-3 human prostate tumour xenografts. CB 1954 (25 mg/kg) was given i.p. daily (×4) NRH (t) followed after 30 min by either 250 mg/kg of NRH (*****)or EP-0152R (♦) given i.v. Neither CB 1954, NRH alone nor EP-0152R alone showed any significant difference from the untreated control (not shown).

NITROREDUCTASE ENZYMES FOR GENE-DIRECTED ENZYME PRODRUG THERAPY GDEPT

A gene therapy-based approach for targeting cancer cells and making them sensitive to CB 1954 has been proposed and is now in clinical trial [64]. GDEPT (gene-directed enzyme prodrug therapy, (Fig. **7**) is used to express an *E. coli* nitroreductase in tumour cells [65–69]. This bacterial enzyme can bioactivate CB 1954 much more efficiently than rat NQO1 [70,71], and has similar activity to NQO2 but can utilise the biogenic co-factors, NADH or NADPH. Human tumour cells transduced to express this enzyme are very sensitive to CB 1954 [65–69]. The nitroreductase enzyme could also be targeted using a tumour-localising antibody or ADEPT (antibody-directed enzyme prodrug therapy) strategy (reviewed in [72]). The advantage of this approach is that it does not rely on endogenous expression of a nitroreductase such as NQO1 or NQO2, and is therefore applicable to many tumour types.

In common with NQO1 and NQO2, the FMN-dependent nitroreductase from *E. coli* B reduces quinones (such as menadione) to their corresponding hydroquinones, and similarly, its activity is inhibited by dicoumarol. Furthermore, the enzyme is also a potential bioreductive activator of certain nitro-containing prodrugs to cytotoxic anti-tumour agents. CB 1954 is activated by nitroreductase under aerobic conditions to a potent difunctional alkylating agent. However, in contrast to NQO1 and NQO2, which generate only the 4-hydroxylamine product, the *E coli* enzyme generates equimolar amounts of the 2- and 4-*mono*hydroxylamino products but does not reduce both nitro groups (Knox *et al*., 1992). It is also a more active enzyme towards CB 1954 than NQO1, with a k_{cat} value of 360 min^{-1} (see Table **1**).

The nitroreductase enzyme belongs to a structurally homologous family of flavoproteins comprising two sub-groups, namely the flavin reductases, and nitroreductases, of which the *E. coli* B nitroreductase is a member. There is little sequence or known structural homology of this novel group with other flavoproteins. In common with the "classical nitroreductase" of *Salmonella typhimurium*, the *E. coli* B enzyme is capable of reducing nitrofurazone. The

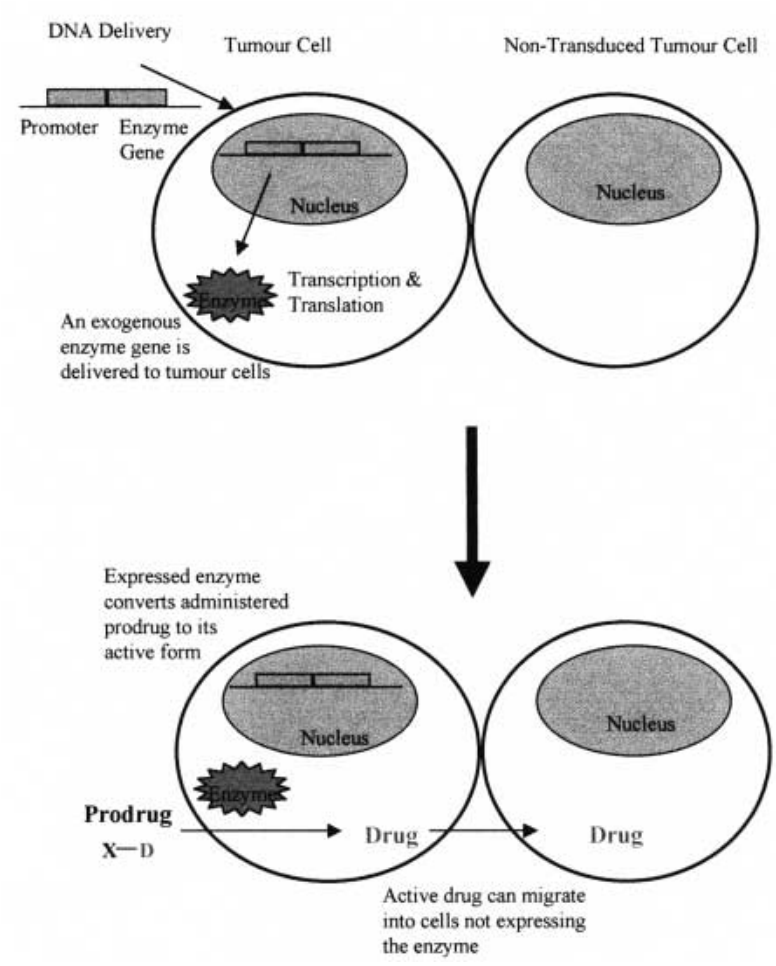

Fig. (7). The generation of a cytotoxic drug by GDEPT. In the first phase, the cell is transduced with gene coding for a prodrug-activating enzyme. This enzyme is expressed and a prodrug is administered which is converted to an active drug (**D**) by the bound enzyme. Importantly, the active drug can migrate and have cytotoxic effects on cells that have not been transduced.

X-ray crystal structure of nitroreductase has recently been determined and analysed in terms of structure and mechanism in comparison with NQO1 [73]. There are a number of significant differences, notably for the substrate-binding pockets that differ substantially in conformation. Overlay of the structural portions of the ligand-binding pockets for the two enzymes reveals that only the *re* face of the FMN co-factor is accessible in nitroreductase whereas, in contrast, it is the *si* face of FAD that can be accessed in NQO1. It is notable that the enzymes despite the marked structural divergence share similar substrates and inhibitors.

Although *E. coli* nitroreductase requires a co-substrate to provide a source of reducing equivalents, this is not a problem in GDEPT as the enzyme is expressed intracellularly, so that the endogenous co-factors NADH or NADPH (nitroreductase can use either [70]) can be exploited. In fact, the co-factor requirement may be an advantage since any enzyme that escapes into the circulation (e.g., from dying cells) will be incapable of activating circulating prodrug due to the lack of a co-factor. This is because NAD(P)H is very rapidly metabolised by serum components [74]. A recombinant retrovirus encoding the nitroreductase has been used to infect mammalian cells; the resulting NIH3T3 cells expressing NR could then be killed with CB 1954. The bulk infected, unselected cell population was ~100-fold more sensitive to CB 1954 than the parental cells [65]. A selected clone was even more sensitive and, using a cell count assay, was >1000-fold more sensitive to CB 1954 than parental NIH3T3 cells (Fig. **8**) [65]. Similar results were found with human melanoma, ovarian carcinoma and mesothelioma cells [68]. The rapid action of CB 1954 [75] and the resulting need for a shorter exposure time may actually facilitate the clinical use of this prodrug. A significant bystander effect was observed and admixed, unmodified NIH3T3 cells could also be killed by a normally non-toxic dose of prodrug [65]. The bystander effect is mitigated by diffusible metabolites, and both the 2- and 4-hydroxylamine derivatives of CB 1954 are released into the medium by CB 1954-treated nitroreductase-expressing NIH3T3 cells [67]. Importantly, and in contrast to the HSV-tk/ganciclovir enzyme/prodrug system, cell killing by nitroreductase/CB 1954 was cytotoxic towards non-cycling cells [65]. A preliminary investigation of nitroreductase/CB 1954 for the treatment of tumours *in vivo* resulted in a regression of tumours expressing nitroreductase following administration of CB 1954, with a significantly increased median survival [69].

Alternative potential prodrugs for use with the nitroreductase system have been proposed. Chinese hamster V79 cells transfected with a nitroreductase expression vector were 770-fold more sensitive to CB 1954 than non-expressing control cells. Other prodrugs such as nitrofurazone (97-fold) and the antibiotic nitroimidazole compounds, misonidazole (21-fold) and metronidazole (50-fold), also showed increased cytotoxicity against the nitroreductase-expressing cells and were found by HPLC to act as substrates for the purified enzyme [66]. However, this correlation was not absolute; in particular the quinone EO9 [3-hydroxymethyl-5-aziridinyl-1-methyl-2-(*H*-indole-4,7-dione)-propenol] showed only a small <3-fold differential. This is probably because this agent can also undergo activation by endogenous enzymes such as

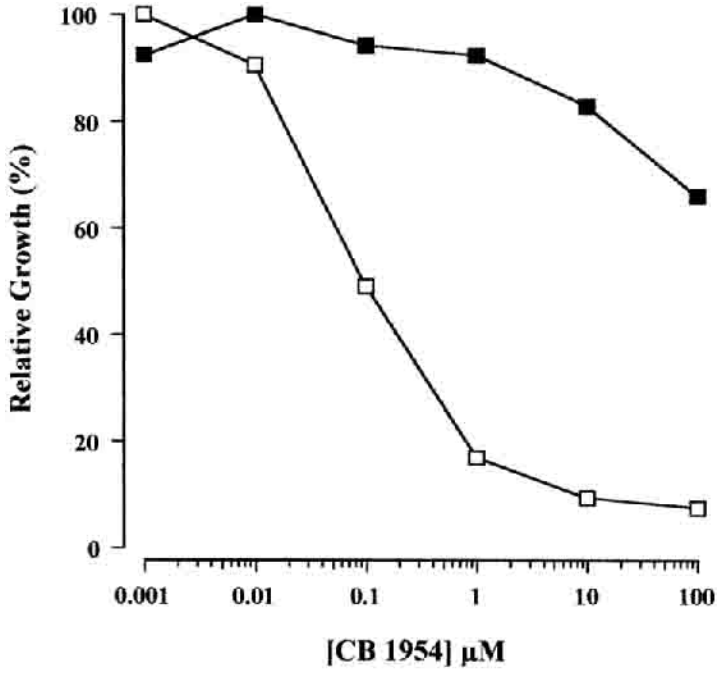

Fig. (8). The cytotoxicity of CB 1954 against an *E. coli* B nitroreductase-expressing cell line. NIH3T3 cells were infected with a recombinant retrovirus containing nitroreductase and a cell clone (NIH3T3-NR) derived by limiting dilution. Parental NIH3T3 cells (n) or NIH3T3-NR cells (q) were treated with CB 1954 for 24 h prior to assay. Adapted from ref. [65].

NADPH:cytochrome P450 reductase [66]. In contrast, extracellular activation of misonidazole or metronidazole by nitroreductase results in little enhancement of cytotoxicity [76], suggesting that the active species have only a very short half-life and probably do not exhibit a large bystander effect. A series of 2,4-dinitrobenzamide mustard analogues of CB 1954 have also been evaluated as potential prodrugs [76]. Other potential prodrugs that could be used in GDEPT are those activated by a self-immolative mechanism (Fig. **9**) that can potentially generate active drugs such as mustards, actinomycins or mitomycin C [77], enediynes [78], *seco*-CI alkylating agents [79] or tallimustine [80]. In each case, the prodrugs are 4-nitrobenzyloxycarbonyl derivatives of these agents which, upon enzymatic reduction, release the active drug species through self-immolation of the formed 4-(hydroxylamino)benzyloxycarbonyl intermediate.

The *in situ* generation of an alkylating agent probably offers the most potent means of killing targeted cell types. However, given the disparate modes of action of the various activating systems available (e.g., nitroreductase/CB 1954 or HSV-tk/ganciclovir), a combination of these approaches offers a means to derive potentially synergistic effects. Thus, co-operative cell killing has been reported for cells expressing *both* nitroreductase and HSV-tk upon treatment with a combination of CB 1954 and ganciclovir [65].

On this basis, CB 1954 is a good example of the requirements of an ideal prodrug for use in GDEPT when activated by nitroreductase, and this enzyme can also be used in combination with other prodrugs. However, systemic administration of the present generation of gene therapy vectors is not possible and GDEPT, unlike ADEPT, is thus limited to isolable tumour deposits, such as intracerebral tumours [81] or prostate cancers [82] that are surrounded by largely non-dividing normal tissue. In such cases, GDEPT is feasible using retrovirus-based vectors. Improved vectors have been proposed and are certainly under development. However, it should be considered that GDEPT is only one answer to certain limitations of the current gene therapy vectors and that a perfect, tumour-specific gene delivery system would ultimately make GDEPT obsolete [83].

OTHER TARGETING STRATEGIES

An alternative approach to GDEPT that delivers the actual active enzyme, rather than the DNA encoding for it, has been termed ADEPT (antibody-directed enzyme prodrug 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 tumour associated antibody (this can be done either chemically or by using recombinant DNA techniques) and allowed to localise to the tumour [84-87]. Thus, ADEPT creates a tumour environment with a high concentration of an enzyme that would convert a normally inert substrate to a highly reactive metabolite. The targeted antibody-enzyme conjugate is designed to remain extra-cellular, as internalised conjugate would be expected to be rapidly degraded in the lysosomal compartment. A number of enzymes and prodrugs have that have been considered for ADEPT and have been reviewed in detail elsewhere [88-91]. A polymer-based system for targeting enzymes to tumours that is directly analogous to ADEPT is called MDEPT (macromolecular directed enzyme prodrug therapy) [92]. Polymer conjugates, like other molecules with prolonged plasma residence times, can accumulate preferentially in a tumour because of the phenomenon of the enhanced permeability and retention effect [93]. This effect occurs because the physiology of solid tumours differs from that of normal tissues in a number of important aspects, the majority of which stem from differences between the two types of vasculature. Compared with the regular, ordered vasculature of normal tissues, blood vessels in tumours are often highly abnormal, distended capillaries with leaky walls, sluggish flow and enhanced permeability to macromolecules [94].

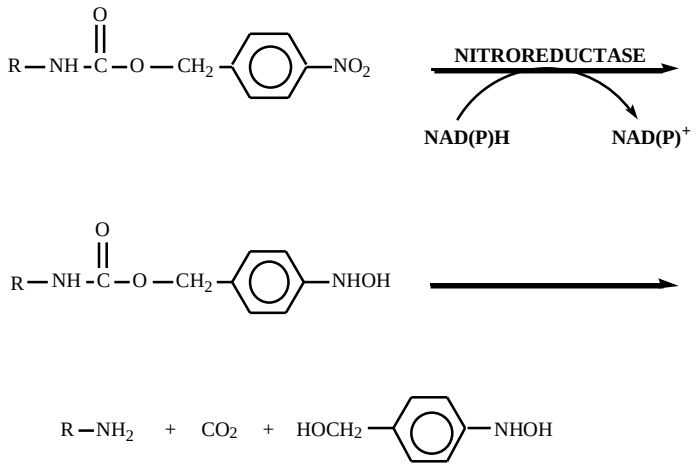

Fig. (9). Generation of an active amino-drug (R-NH_2) by self-immolation of a prodrug following nitroreduction. By analogy with these carbamates, analogous carbonate prodrugs can also undergo reduction and self-immolation to release an active drug (i.e., R-OH)

The use of nitroreductase enzymes in these approaches is limited by the fact that they require a co-substrate to act as a source of reducing equivalents. Further both NADH and NAPH are very rapidly metabolised in serum so they could not be co-administered with the prodrug [74]. However, it has been found that both NQO1 and NR do not require the complete NAD(P)H structure for co-substrate activity and a synthetic 'virtual co-factor' suitable for applications such as ADEPT has been described [95]. This compound, nicotinic acid riboside (reduced), is, unlike NADH, stable to metabolism by serum enzymes [95].

For NQO2, the use of permeable co-substrates allows the direct use of prodrug therapy without the complications associated with macromolecular targeting systems. However, 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyridine is a good co-substrate for NQO2. The compound is negatively charged at pH7 and this charge prevents it from entering cells [59]. Use of a charged co-substrate of this type would allow NQO2 to be used in targeted therapies such as ADEPT. As the targeted NQO2 would be external, the co-substrate would allow the tumour-specific bioactivation of CB 1954 but would not facilitate activation by the endogenous enzyme [59].

These results demonstrate that the requirement for a cofactor need not be a limitation in the use of reductive enzymes in ADEPT or MDEPT.

CONCLUSIONS AND FUTURE POTENTIAL

The emergence of nitroreduction strategies based on NQO1, NQO2, GDEPT, ADEPT or MDEPT for cancer treatment presents an exciting opportunity for new developments in tumour-targeted medicinal chemistry. A critical requirement for anti-tumour drugs, often neglected and responsible for their clinical failure, is the selective targeting of tumour cells without adverse effect upon normal tissue [20,72,96,97]. Exploitation of endogenous enzymes associated with tumours, or vector-assisted delivery to tumours, offers a potential route to genuinely selective and effective prodrugs with viable clinical application. The recent availability of high-quality crystal structures for the flavoprotein enzymes NQO1, NQO2 and nitroreductase offers detailed information at the molecular level for use in the design of improved prodrugs and/or co-factors (co-substrates). Systematic evaluation of candidate enzyme–drug/co-factor models will improve our understanding of the key events involved in bioreductive activation. Considerable success has been achieved with the development of highly selective bioreduction systems, particularly through control of the biochemical requirements for a non-endogenous co-factor in the case of NQO2 and its activation of the archetypal CB 1954 prodrug. While this has largely stemmed from conventional medicinal chemistry approaches, it is clear that rational structure-based methods could present alternative or superior molecules. With respect to human NQO1, the rational design of a CB 1954 analogue that is selectively activated by NQO1 but at a rate sufficient to give anti-tumour activity may be achievable. Self-immolative molecules, which have been described for nitroreductase, offer an attractive approach for prodrug development as they can release a range of different active molecules with various mechanisms of action from the same basis prodrug molecule [77]. An alternative approach would be to use structure-based methods to design new proteins with the required structure activity-relationship for use in ADEPT and GDEPT.

In summary, aerobic nitroreduction can be exploited for the selective activation of prodrugs at a tumour. When combined with the knowledge of the structure of the relevant flavoenzymes this presents an enticing chance for rational new developments in medicinal chemistry.

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ABBREVIATIONS

ADEPT	=	Antibody-directed enzyme prodrug therapy
CB 1954	=	5-(1-Aziridinyl)-2,4-dinitrobenzamide
FAD	=	Flavin adenine dinucleotide
FMN	=	Flavin mononucleotide
GDEPT	=	Gene-directed enzyme prodrug therapy
MDEPT	=	Macromolecule-directed enzyme prodrug therapy
NMOR	=	NAD(P)H menadione oxidoreductase
NQO1	=	NAD(P)H:quinone oxidoreductase 1 [DT-diaphorase, (EC 1.6.99.2)]
NQO2	=	NAD(P)H:quinone oxidoreductase 2
NRH	=	Dihydronicotinamide riboside [nicotinamide riboside(reduced)]

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