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VIRTUAL COFACTORS FOR AN *ESCHERICHIA COLI* NITROREDUCTASE ENZYME: RELEVANCE TO REDUCTIVELY ACTIVATED PRODRUGS IN ANTIBODY DIRECTED ENZYME PRODRUG THERAPY (ADEPT)

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Abstract—A nitroreductase enzyme has been isolated from *Escherichia coli* that has the unusual property of being equally capable of using either NADH or NADPH as a cofactor for the reduction of its substrates which include menadione as well as 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954). This property is shared with the mammalian enzyme, DT diaphorase. The nitroreductase can, like DT diaphorase, also use simple reduced pyridinium compounds as virtual cofactors. The intact NAD(P)H molecule is not required and the simplest quaternary (and therefore reducible) derivative of nicotinamide, 1-methylnicotinamide (reduced), is as effective as NAD(P)H in its ability to act as an electron donor for the nitroreductase. The structure–activity relationship is not identical to that of DT diaphorase and nicotinic acid riboside (reduced) is selective, being active only for the nitroreductase. Irrespective of the virtual cofactor used, the nitroreductase formed the same reduction products of CB 1954 (the 2- and 4-hydroxylamino derivatives in equal proportions). Nicotinic acid riboside (reduced), unlike NADH, was stable to metabolism by serum enzymes and had a plasma half-life of seven minutes in the mouse after an i.v. bolus administration. NADH had an unmeasurably short half-life. Nicotinic acid riboside (reduced) could also be produced *in vivo* by administration of nicotinic acid 5′-*O*-benzoyl riboside (reduced). These results demonstrate that the requirement for a cofactor need not be a limitation in the use of reductive enzymes in antibody directed enzyme prodrug therapy (ADEPT). It is proposed that the *E. coli* nitroreductase would be a suitable enzyme for ADEPT in combination with CB 1954 and a synthetic, enzyme-selective, virtual cofactor such as nicotinic acid riboside (reduced).

Key words: nitroreduction; CB 1954; prodrugs; NADH; NADPH; bioreduction; bioactivation

An innovation in anticancer chemotherapy has been the ADEPT§ approach [1–3] which is an attempt to produce a tumour-selective cytotoxicity. A tumour-selective monoclonal antibody (or fragment) is conjugated to an enzyme that is capable of bioactivating a prodrug. The antibody is allowed to localize at the site of the tumour and the unlocalized conjugate is given time to be eliminated from the body (or is hastened by a 'clearance' antibody). A non-toxic prodrug is then administered. This can only be activated at the site of the tumour by the bound conjugate [1–3]. A number of potential enzyme–prodrug combinations have been proposed. The enzymes carboxypeptidase G2 [1–4], penicillin amidase [5], alkaline phosphatase [6], β-lactamase [7, 8] and cytosine deaminase [9] have all been considered. These can activate various prodrugs by hydrolysis.

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§ Abbreviations: ADEPT, antibody directed enzyme prodrug therapy; CB 1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; NRH, nicotinamide riboside (reduced); NARH, nicotinic acid riboside (reduced); FCS, foetal calf serum; PBS, phosphate-buffered saline.

Nitroreduction is also capable of activating prodrugs. For example, it can activate compounds based on CB 1954 [10, 11]. The basis of this activation is that CB 1954 (a monofunctional weak alkylating agent) is reduced to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a difunctional strong alkylating agent [10]. This bioactivation has been fully elucidated. Briefly, after reduction, the hydroxylamine undergoes a further chemical activation by reaction with a thioester, to form the ultimate DNA–DNA crosslinking species (probably 4-(acetoxyamino)-5-(aziridin-1-yl)-2-nitrobenzamide) [12]. In rat cells this reduction is performed aerobically by the enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2) [11]. The DNA crosslinks induced by CB 1954 are formed at a very high frequency (>70%) with respect to total DNA adducts and they are also poorly repaired [13]. These unusual properties of the crosslink may explain the large increase in cytotoxicity accompanying the bioactivation that can be about 10,000-fold on a dose basis. So large an increase in cytotoxicity makes CB 1954 an attractive candidate prodrug for ADEPT.

It is a requirement of ADEPT enzymes that an equivalent activity does not exist within the host's cells. Despite being a mammalian enzyme, rat DT diaphorase may be suitable in this respect, because

the human form of DT diaphorase is much less able to metabolize CB 1954 than is the rat form [14]. Further, a nitroreductase enzyme has recently been isolated from *E. coli*. This can also bioactivate CB 1954 but reduces the drug about 100-fold faster than does rat DT diaphorase [15, 16]. Thus, CB 1954 in conjunction with this nitroreductase is potentially an attractive ADEPT system.

However, in the above combination a cofactor would also have to be present to supply a source of reducing equivalents for the enzyme. The biogenic cofactors for reductases (NADH and NADPH) are not suitable, being very rapidly oxidized by serum proteins and on the cell surface [17].

The *E. coli* nitroreductase and DT diaphorase share the unusual property of being able to use either NADH or NADPH as a cofactor for the reduction of their substrates [15]. For DT diaphorase, it has been shown that the intact NAD(P)H molecule is not required. Other reduced pyridinium compounds can also act as cofactors for DT diaphorase and some simple quaternary (and therefore reducible) derivatives of nicotinamide are effective sources of reducing equivalents for this enzyme [18].

We now report on the ability of the *E. coli* nitroreductase to use synthetic reduced pyridinium derivatives as virtual cofactors and on the pharmacokinetics of a compound that may be suitable for applications in ADEPT.

MATERIALS AND METHODS

Materials. All biochemical reagents were supplied by Sigma (Poole, U.K.) unless otherwise stated.

Purification of the nitroreductase enzyme from E. Coli *B.* The enzyme was purified as previously described [15].

Synthesis of pyridinium derivatives. NADH (**1**, Fig. 1), NADPH (**2**, Fig. 1), nicotinamide ribotide (nicotinamide mononucleotide) (reduced form) (**8**, Fig. 1), α-NADH (**3**, Fig. 1), α-NADPH (**4**, Fig. 1), 3-acetylpyridineadenine dinucleotide (reduced form) (**5**, Fig. 1), nicotinic acid ribotide (nicotinic acid mononucleotide) (**10** (reduced), Fig. 1) and 1-methylnicotinamide (**14** (reduced), Fig. 1) were supplied by Sigma (Poole, U.K.). Nicotinamide riboside (**9** (reduced), Fig. 1) was prepared from the ribotide by the action of the enzyme alkaline phosphatase as previously described [18]. 3-Acetylpyridine ribotide (reduced form) (**6**, Fig. 1) was prepared by cleaving 3-acetylpyridineadenine dinucleotide (reduced form) (**5**, Fig. 1) with phosphodiesterase again as previously described [18]. 3-Acetylpyridine riboside (reduced form) (**7**, Fig. 1) was prepared from the ribotide as described for nicotinamide riboside above. Nicotinamide 5′-*O*-benzoyl riboside (**15** (reduced), Fig. 1) was synthesized as described by Jarman and Ross [19]. 4-Methyl nicotinic acid 5′-*O*-benzoyl riboside (**16** (reduced), Fig. 1) and 4-methyl nicotinic acid riboside (**17** (reduced), Fig. 1) were both synthesized as previously described [18, 20], as was nicotinic acid riboside (**11** (reduced), Fig. 1) and nicotinic acid 5′-*O*-benzoyl riboside (**12** (reduced), Fig. 1) [18].

Synthesis of the previously unreported compound 1-β-D-ribofuranosyl-3-(5-tetrazolyl)pyridine (**13** (reduced), Fig. 1) is described below.

A suspension of 5-(3-pyridyl)-tetrazole (Maybridge Chemical Co. Ltd, Tintagel, Cornwall, U.K.) (0.22 g, 1.5 mmol) and 3,5-di-*O*-benzoylribosyl chloride (synthesized as previously described [21] (0.29 g, 0.75 mmol) in dry acetonitrile (37.5 mL) was stirred at room temperature for 2 days. The solution was filtered, concentrated to 10 mL, diluted with ethyl acetate (50 mL) filtered again and applied to a preparative HPLC column (160 g Merck Kiesgel 60; Art 11695), contained in an Axxial Chromatospac Prep 10, which was eluted with ethyl acetate/ethanol (3:2). The fractions containing product were evaporated to dryness and hydrolysed without further purification. (Attempts at recrystallization caused decomposition.) The residue was dissolved in methanol (10 mL), cooled to −18°, and a solution of ammonia (2N) in methanol (0.25 mL) was added. Two weeks later the supernatant was decanted, the solid washed with methanol and dried to give 81 mg (39%) of brown crystals, m.p. 180–183° (decomp.). ^{1}H NMR (Bruker AC Spectrometer, 250 MHz, solution in d_6-DMSO): δ 9.60 (1H, s, 2-H), 9.11 (1H, d, J = 6 Hz, 4 or 6-CH), 9.05 (1H, dd, J = 1.5 Hz and 8 Hz, 4 or 6-CH), 8.22 (1H, dd, J = 6 Hz and 8 Hz, 5-CH), 6.20 (1H, d, J = 5 Hz, 1′-CH), 5.85 (1H, d, J = 6 Hz, 2′-OH), 5.36 (1H, d, J = 5 Hz, 3′-OH), 5.29 (1H, t, J = 5 Hz, 5′-OH), 4.3 (2H, m, 2′ + 4′-CH), 4.15 (1H, q, J = 5 Hz, 3′-CH), 3.82 (1H, dt, $J_{5',5'}$ = 12 Hz, $J_{4',5'}$ = 3 Hz, $J_{5',5'\text{-OH}}$ = 5 Hz, 5′-CH_2), 3.72 (1H, dt, $J_{5',5'}$ = 12 Hz, $J_{4',5'}$ = 3 Hz, $J_{5',5'\text{-OH}}$ = 5 Hz, 5′-CH_2). Mass spectrometry was carried out on a TSQ 700 triple–quadrupole system (Finnigan MAT) equipped with an electrospray ion source (Analytica): m/z 280 $[M + H]^+$, 148 [M-ribose + H]$^+$. Analysed: C, H, N; Calculated for $C_{11}H_{13}N_5O_4$ = C 47.31; H 4.69; N 25.08. Found: C 47.02; H 4.57; N 25.02.

Chemical reduction of synthetic cofactors. Chemical reduction was as previously reported [18]. The concentration of the purified reduced product was calculated assuming an ε_{340} of 6200 (i.e. the same as NAD(P)H).

Enzyme activity and kinetic studies. The nitroreductase enzyme was assayed as previously described for DT diaphorase, employing either menadione as the substrate and cytochrome *c* as terminal electron acceptor [11] or CB 1954 as substrate [14, 15]. K_m values were determined from Eadie–Hofstee plots. Standard deviations of these determinations were <10%.

Cofactor pharmacokinetics in the mouse. NADH (**1**, Fig. 1), nicotinic acid 5′-*O*-benzoyl riboside (reduced) (sodium salt) (**12**, Fig. 1) or NARH (**11**, Fig. 1) was dissolved at 100 mM in PBS. Female BalbC mice were injected intravenously with either NADH, nicotinic acid 5′-*O*-benzoyl riboside (reduced) or NARH at 10 mL/kg. At various times following injection, mice were anaesthetized with halothane and plasma samples were prepared by centrifugation of intracardiac blood collected into iced heparinized tubes. The plasma was subjected to centrifugal ultrafiltration (Millipore, Ultrafree-10) at 10,000 *g*. The ultrafiltrate was then analysed

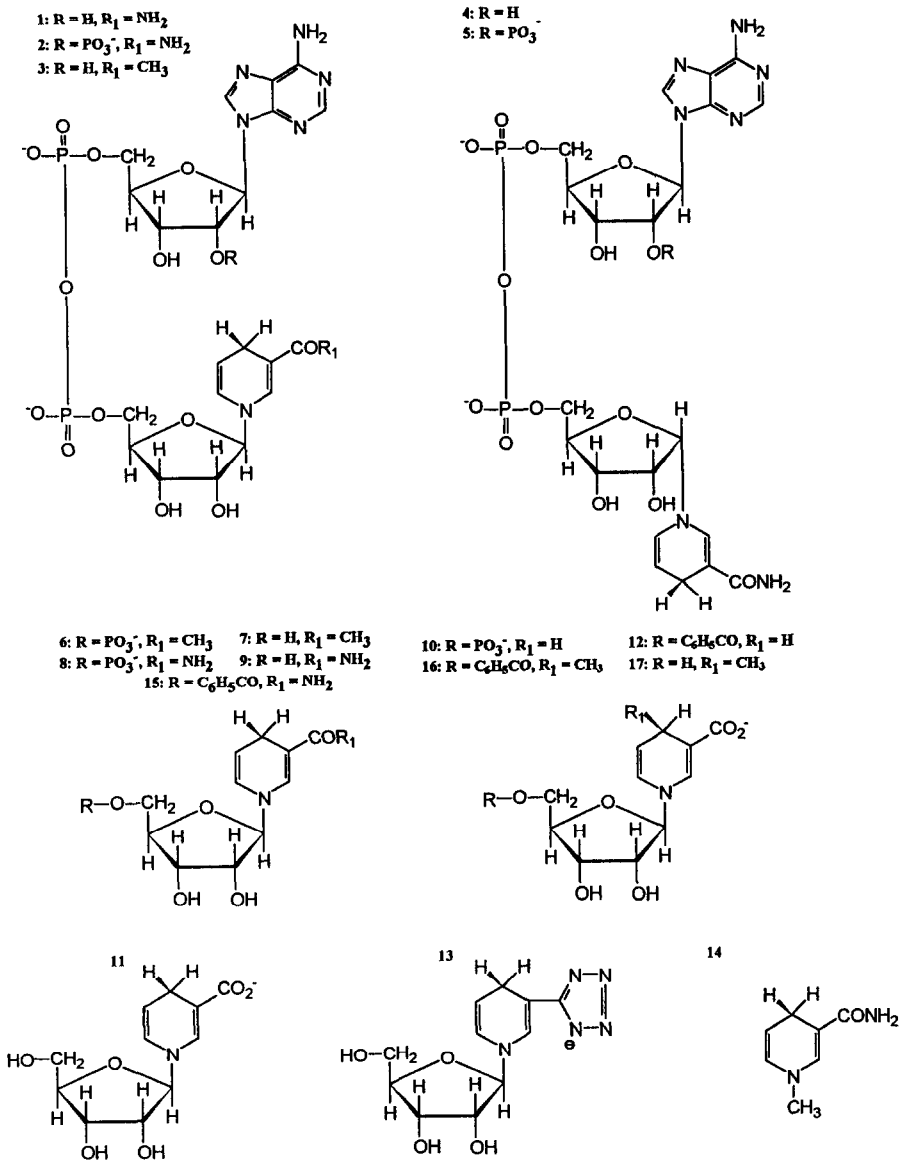

Fig. 1. The structures of the pyridinium compounds used in this study. The numbers refer to the compound as listed in Table 1. All compounds are illustrated in their active, reduced, form.

for cofactor concentration by HPLC and quantified by fluorescence [17, 18].

RESULTS

The cofactor specificity of the nitroreductase

The ability of various reduced quaternary pyridinium derivatives to act as cofactors for the nitroreductase has been measured using either CB 1954 or menadione as substrate. The relative reduction of CB 1954 and the K_m values (determined against menadione) for these compounds are shown in Table 1. The K_m values were determined using menadione. This was because, as we have previously shown, the calculated K_m of NADH decreases with decreasing CB 1954 concentration (suggesting a substituted enzyme mechanism) [15]. Using the menadione assay, the substrate concentration is low (10 μM) and is constant during the assay (because cytochrome *c* is used as the final electron acceptor). All cofactors gave the same K_{cat} of 4.2×10^4 as previously reported for NADH (**1**, Fig. 1) [15].

None of the oxidized forms of these compounds had any activity in either assay nor did any interaction occur between the substrate and the cofactor in the absence of enzyme. Only approximate data could be obtained for nicotinic acid ribotide (reduced) (**10**, Fig. 1) and 1-methylnicotinamide (reduced) (**14**, Fig. 1) because of the rapid spontaneous oxidation of their reduced forms at neutral pH. However, both compounds can act as a cofactor for the nitroreductase enzyme. The reduced forms of 4-methylnicotinic

Table 1. The ability of various quaternary pyridinium analogues to act as virtual cofactors for the *E. coli* nitroreductase*

Number	Compound	Relative rate of CB 1954 reduction	K_m (μM)
1	NADH	1.0	<10
2	NADPH	1.0	<10
3	α-NADH	0.24	246
4	α-NADPH	0.46	114
5	3-Acetylpyridine-adenine dinucleotide	0.86	24
6	3-Acetylpyridine-ribotide	0.97	125
7	3-Acetylpyridine-riboside	<0.01	ND
8	Nicotinamide ribotide	0.94	115
9	Nicotinamide riboside (NRH)	<0.01	ND
10	Nicotinic acid ribotide	0.98	<10
11	Nicotinic acid riboside (NARH)	1.02	97
12	Nicotinic acid 5′-*O*-benzoyl riboside	0.32	<10
13	1-β-D-Ribofuranosyl-3-(5-tetrazolyl) pyridine	<0.01	ND
14	1-Methylnicotinamide	~1.0	~50
15	Nicotinamide 5′-*O*-benzoyl riboside	<0.01	ND
16	4-Methylnicotinic acid-5′-*O*-benzoyl riboside	<0.01	ND
17	4-Methylnicotinic acid-riboside	<0.01	ND

* All compounds were used in their reduced forms, the oxidized forms having no activity. The relative rates were measured at initial concentrations of cofactor and CB 1954 and 500 μM and 100 μM, respectively. Using NADH, the actual rate of CB 1954 reduction was 2 μmol/min/mg enzyme. The K_m values were determined using menadione (10 μM) as a substrate.

acid 5′-*O*-benzoyl riboside (**16**, Fig. 1) and 4-methylnicotinic acid riboside (**17**, Fig. 1) are chiral. Whilst the two reduced enantiomers of 4-methylnicotinic acid 5′-*O*-benzoyl riboside (**16**, Fig. 1) could not be resolved, those of 4-methylnicotinic acid riboside (reduced) (**17**, Fig. 1) could. However, neither the racemate nor the resolved enantiomers showed any activity with the nitroreductase.

In general, the enzyme shows little requirement for the adenosine portion of NAD(P)H with the reduced ribotide derivatives showing similar activity to the intact molecule (**6, 8, 10**, Fig. 1). However, removal of the 5′-phosphate group, to produce the reduced riboside, destroyed the ability of the molecule to act as a cofactor (**7, 9, 13–17**, Fig. 1). The exception was NARH (**11**, Fig. 1) which retained full cofactor activity but with a decreased affinity of the enzyme for the cofactor. The tetrazole group is generally a good acid mimic [22] and has a similar pK_a to the carboxyl group. Therefore the novel compound 1-β-D-ribofuranosyl-3-(5-tetrazolyl)-pyridine (reduced) (**13**, Fig. 1) was synthesized. However, this compound showed a total lack of cofactor activity. The same loss of activity was also observed with any substitution of a methyl group on the 4-position of any reduced nicotinic acid derivative (**16, 17**, Fig. 1). Surprisingly, whilst reduced ribotides tended to be active and reduced ribosides not, the ribose moiety can be dispensed with and 1-methylnicotinamide (reduced) (**14**, Fig. 1) is a good cofactor for nitroreductase. This compound is the simplest quaternary (and therefore reducible) nicotinamide analogue.

1-β-D-Ribofuranosyl-3-(5-tetrazolyl)pyridine (reduced) (**13**, Fig. 1) is a previously unreported compound and was also tested against DT diaphorase isolated from Walker tumour cells. Like nitroreductase, DT diaphorase could not use this compound as a cofactor substitute to reduce either menadione or CB 1954.

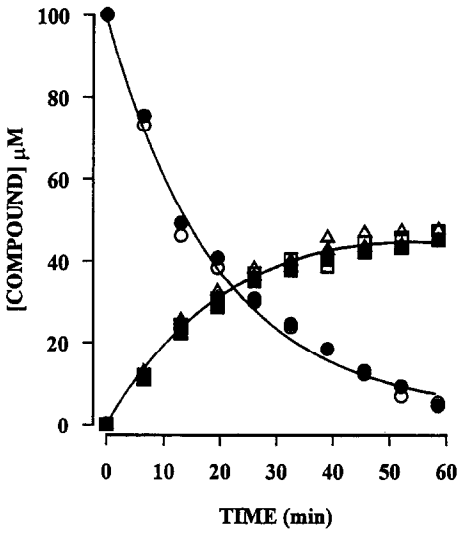

Fig. 2. The aerobic reduction of CB 1954 (○) and the resulting formation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (■) and 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (▲) by the *E. coli* nitroreductase using either NADH (solid symbols) or NARH (open symbols) as a cofactor. The enzyme concentration was 2 μg/mL and the initial cofactor concentration was 500 μM.

The products of the enzymatic reduction of CB 1954

Using NADH as cofactor, reduction of CB 1954

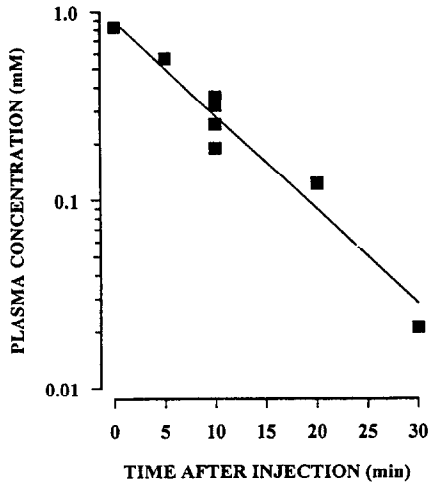

Fig. 3. The pharmacokinetics of nicotinic acid riboside (reduced) (NARH) in mouse plasma after i.v. injection. Each symbol represents an individual mouse. The half-life is 7 min.

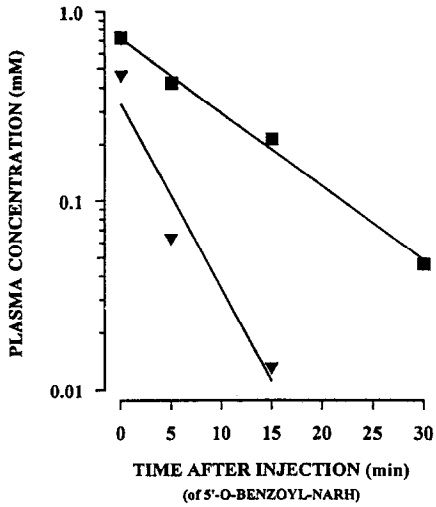

Fig. 4. The formation and pharmacokinetics of NARH in mouse plasma after i.v. injection of nicotinic acid 5′-*O*-benzoyl riboside (reduced). After injection there is an immediate formation of NARH (■) with little nicotinic acid 5′-*O*-benzoyl riboside (reduced) (▼) persisting. Each time-point represents an individual mouse. The half-life of the NARH so formed is 7.7 min.

by the *E. coli* nitroreductase resulted in the formation of two products (Fig. 2). By comparison of retention times and spectral characteristics (determined using a diode array detector) with known standards, these products have previously been shown to be 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide [16]. Reduction of CB 1954, using the other compounds that had been shown to be able to act like cofactors, also produced the same two products at the same relative rates. This is illustrated in Fig. 2 using NARH (**11**, Fig. 1). No other CB 1954 metabolites were found.

The pharmacokinetics of NADH and NARH in the mouse

After i.v. injection, exponential decay in the concentration of NARH (**11**, Fig. 1) was observed (Fig. 3). Regression analysis gave a half-life of 7 min. In contrast, no NADH was detectable after the initial injection, indicating a half-life of <1 min (data not shown). Injection of nicotinic acid 5′-*O*-benzoyl riboside (reduced) resulted in the immediate formation of NARH with only a little amount of the parent being observed (Fig. 4). The half-life of the NARH so generated was similar (7.7 min) to that observed with direct injection of this compound (Fig. 4).

DISCUSSION

We have shown that a nitroreductase enzyme isolated from *E. coli* can reduce CB 1954 to its cytotoxic 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide derivative and suggested that this enzyme might be suitable for ADEPT [15, 16]. For a nitroreductase enzyme to be used in ADEPT a cofactor would also have to be administered to supply a source of reducing equivalents. The biogenic cofactors for reductases, NADH and NADPH, are not suitable, being far too rapidly oxidized by serum proteins and cell surfaces [17]. An alternative approach is the development of a synthetic cofactor that will have the required pharmacokinetic properties.

Although there is no sequence homology between the two enzymes, the *E. coli* nitroreductase shares some similar biochemical properties with rat DT diaphorase [15, 16]. For example, both enzymes can reduce quinones and are inhibited by dicoumarol. Importantly, both enzymes can reduce CB 1954 aerobically, using either NADH or NADPH as a cofactor. Most enzymes can distinguish between NADPH and NADH and can use only one or the other and this is a reflection of the fundamental distinction made between these two cofactors in biochemical reactions. In the case of DT diaphorase, the intact NAD(P)H molecule is not required and simple quaternary (and therefore reducible) derivatives of nicotinamide (such as 1-methylnicotinamide (reduced)) are virtual cofactors for this enzyme [18].

We have now shown that, for both the nitroreductase and DT diaphorase, the actual structural requirements for a virtual cofactor are fairly lax. There is little requirement for the adenine nucleotide portion of NAD(P)H. The simplest quaternary (thus reducible) derivative of nicotinamide, 1-methylnicotinamide (reduced), was as good a cofactor as NAD(P)H. However, the cofactor requirements of the nitroreductase and DT diaphorase are not identical. For example, nicotinic acid riboside (reduced) (NARH) is an excellent cofactor for the nitroreductase but is not a cofactor for DT diaphorase. Conversely, nicotinamide

riboside (reduced) (NRH) is a cofactor for DT diaphorase but not nitroreductase. Thus the acid moiety has introduced selective specificity between the two enzymes. However, the carboxyl group could not be substituted by a tetrazole group even although it is often a good acid mimic [22]. It is of interest in this context that 5-(3-pyridyl)tetrazoles have been investigated as mimics of lipolysis-inhibiting nicotinic acids. They were found to be much less potent inhibitors *in vitro* than the equivalent nicotinic acids [23].

The ability to have enzyme selectivity with these compounds is very important with respect to the use of CB 1954 as a prodrug in ADEPT. We have previously shown that the cytotoxicity of CB 1954 can be potentiated in human cells (normally resistant to CB 1954) by administration of exogenous NADH and that the magnitude of this effect is proportional to the cells' DT diaphorase content [24]. The mechanism of this effect involves the metabolism of NADH by serum proteins to form NRH, which is both permeable to cells and a cofactor for DT diaphorase [24]. For the ADEPT concept to work, it is fundamental that the prodrug is not activated by normal human tissue. It is possible that CB 1954 could be activated by normal tissue (depending on their DT diaphorase levels), by the above mechanism, if cofactors such as NRH or NADH were co-administered. NARH avoids this potential problem. First, it is negatively charged at physiological pH and therefore unlikely to enter a cell. Secondly, it cannot act as a cofactor for DT diaphorase. It can therefore not produce the potentiating effect seen with NRH [24]. Further, its pharmacokinetic properties in the mouse are superior to NADH with a half-life of 7 min after i.v. injection. This is similar to that of the initial distribution kinetics of CB 1954 [25]. NARH can also be rapidly produced *in vivo* by administration of nicotinic acid 5′-*O*-benzoyl riboside (reduced). NARH is formed from this compound presumably by the action of non-specific serum esterases, levels of which are known to be high in mouse serum. As a chemical, nicotinic acid 5′-*O*-benzoyl riboside (reduced) has an advantage over NARH in that it can be prepared in bulk by reverse-phase HPLC without the use of buffer salts. Thus, nicotinic acid 5′-*O*-benzoyl riboside (reduced) may be considered as a 'profactor' of NARH.

In summary, we have shown that the *E. coli* nitroreductase can, like DT diaphorase, use simple synthetic reduced pyridinium compounds as virtual cofactors. However, the structure–activity relationship is not identical to that of DT diaphorase and NARH is selective for the nitroreductase. NARH is, biologically, relatively inert and is pharmacokinetically superior to the biogenic cofactor NADH. These results demonstrate that the requirement for a cofactor need not be a limitation in the use of nitroreductase enzymes in ADEPT. Given the dramatic increase in cytotoxicity upon its bioactivation, CB 1954 and the *E. coli* nitroreductase remains potentially an attractive prodrug/enzyme combination for ADEPT.

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