Biochemical Pharmacology, Vol. 37, No. 24, pp. 4671–4677, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

THE NITROREDUCTASE ENZYME IN WALKER CELLS THAT ACTIVATES 5-(AZIRIDIN-1-YL)-2,4-DINITROBENZAMIDE (CB 1954) TO 5-(AZIRIDIN-1-YL)-4-HYDROXYLAMINO-2-NITROBENZAMIDE IS A FORM OF NAD(P)H DEHYDROGENASE (QUINONE) (EC 1.6.99.2)*

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(*Received* 26 *May* 1988; *accepted* 11 *July* 1988)

Abstract—A nitroreductase enzyme has been isolated from Walker 256 rat carcinoma cells which can convert 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to a cytotoxic DNA interstrand crosslinking agent by reduction of its 4-nitro group to the corresponding hydroxylamino species (Roberts JJ *et al.*, *Biochem Biophys Res Commun* **140**: 1073–1078, 1986; Knox RJ *et al.*, *Biochem Pharmacol* **37**: 4661–4669, 1988). The enzyme has now been identified as a form of NAD(P)H dehydrogenase (quinone) (DT diaphorase, menadione reductase (NMOR), phylloquinone reductase, quinone reductase, EC 1.6.99.2) by comparison of partial protein sequences, coenzymes, substrate and inhibitor specificities, and spectroscopic data. 2-Phenyl-5(4)-aminoimidazole-4(5)-carboxamide and 5(4)-aminoimidazole-4(5)-carboxamide were shown to be inhibitors of the isolated Walker cell enzyme. This observation could explain the reported antagonistic action of the aminoimidazole carboxamides to the antitumour effects of CB 1954.

CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide), a monofunctional alkylating agent, exhibits a highly specific toxicity towards the Walker 256 tumour, a hydrocarbon-induced rat mammary carcinoma, *in vivo* [1]. We showed that the basis of this selective action of CB 1954 on Walker cells is the formation of DNA interstrand crosslinks in these particular cells, but not in non-toxically affected cells [2]. This crosslink formation is a result of the activation of the drug to a difunctionally-reacting species by the action of a nitroreductase enzyme [3]. Thus in Walker cells CB 1954 is reduced to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a difunctional compound which is toxic to cells which are resistant to CB 1954. The enzyme which performs this aerobic nitroreduction has been purified and shown to be a flavoprotein with a molecular weight of 33.5 kDA [2]. We now report the identification of this enzyme as a form of NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2). A number of aminoimidazole carboxamides have been shown to diminish the antitumour effects of CB 1954 [4, 5], 2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide being the most effective [5]. Such observations led to the theory that CB 1954 may act as a purine antimetabolite [4]. We also report that both 5(4)-aminoimidazole-4(5)-carboxamide and 2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide are inhibitors of the isolated enzyme from Walker cells. A similar inhibition *in vivo* could account for the reduction by the aminoimidazole carboxamides of the antitumour effect of CB 1954.

MATERIALS AND METHODS

Materials. All chemicals and reagents were supplied by Sigma (Poole, U.K.) unless otherwise stated. 2-Phenyl-5(4)-aminoimidazole-4(5)-carboxamide was kindly supplied by Dr. P Workman (MRC Clinical Oncology and Radiotherapeutics Unit, Cambridge, U.K.) and 5-chloro-2,4-dinitrobenzamide by Dr. D. Wilman (ICR).

Isolation and purification of a protein with a nitroreductase activity from Walker 256 tumour cells. The purification of the enzyme, by successive gel filtration and anion exchange HPLC, has been previously described [3].

Synthesis of 2,4-dinitro-5-(2'-hydroxyethylamino)benzamide. The compound was prepared by the acid-catalysed hydrolysis of the aziridine ring of CB 1954 [6]. A solution of CB 1954 (100 mg) in DMSO (2 ml) was diluted with 1 M HCl (5 ml). After

* Enzyme nomenclature follows the 1984 recommendations of the Nomenclature Committee of the International Union of Biochemistry. Other names for NAD(P)H dehydrogenase (quinone) [NAD(P)H:(quinone-acceptor) oxidoreductase] (EC 1.6.99.2) include DT diaphorase, menadione reductase (NMOR), phylloquinone reductase, and quinone reductase.

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a few seconds of mixing, a dense bright yellow precipitate of 2,4-dinitro-5-(2′-hydroxyethylamino)-benzamide formed. This was collected by vacuum filtration, washed with distilled water (3 × 2 ml) and dried by vacuum desiccation. The identity of the compound was confirmed by UV-visible spectrophotometry, its spectrum being identical to that of 2,4-dinitro-5-(2′-hydroxyethylamino)benzamide as reported by Ross [6].

Substrate specificity of the nitroreductase enzyme. The ability of the enzyme as isolated from Walker cells to reduce nitro-compounds other than CB 1954 was determined by HPLC using modifications of the isocratic elution conditions used to assay the reduction of CB 1954 [3]. Under these modified conditions the compound of interest was separated from the NADH peak.

For each assay the test substrate (30 μM) was incubated with NADH (500 μM) and enzyme (25 μl, 2 mg/ml) in PBS (500 μl) at 37° and injected onto the HPLC column after various times. The loss of substrate was determined as the decrease in the area of its corresponding peak on the plot of UV absorbance against time.

Alternatively, the reaction of the enzyme with various substrates was monitored spectrophotometrically, by following the decrease in the absorption at 340 nm resulting from the oxidation of NADH.

Enzyme kinetic and inhibition studies. NAD(P)H dehydrogenase (quinone) activities were assayed by a spectrophotometric method similar to that described by Robertson *et al.* [7] using menadione as a substrate and cytochrome *c* as a terminal electron acceptor. Stock solutions of menadione (1 mM), dicoumarol (10 μM) and warfarin (100 mM) were made up in DMSO. All other solutions were made up in 10 mM Tris–HCl buffer, pH 7.5. For K_m and K_i determinations, the final reaction mixture contained menadione (0.0–10.0 μM), cytochrome *c* (70 μM) and NAD(P)H (0.5 mM) in 10 mM Tris–HCl buffer, pH 7.5. Concentrations of various compounds that would inhibit the enzyme, to either 50% or 10% of the control activity, were obtained by including either dicoumarol (0.0–0.1 μM), warfarin (0.0–1.0 mM), CB 1954 (0.0–1.0 mM), 5(4)-aminoimidazole-4(5)-carboxamide (0.0–2.0 mM) or 2-phenyl - 5(4) - aminoimidazole - 4(5) - carboxamide (0.0–2.0 mM) in the reaction mixture. The reaction was started by the addition of reaction mixture (1 ml) to a cuvette containing a volume of enzyme (2.5 μl, 20 μg/ml) and the linear change in absorbance at 550 nm due to the reduction of cytochrome c was monitored over a 75-sec period at 37°. All spectrophotometric measurements were carried out using a Kontron Uvikon 860 UV-visible spectrophotometer. Initial rates of reaction were determined by linear regression analysis ($r > 0.995$). Values of K_m were obtained from Eadie–Hofstee plots and K_i values were derived from Dixon plots. Protein concentration was determined using a conventional protein assay (Bio-Rad) calibrated against bovine serum albumin.

Amino acid sequence analysis. Fragments suitable for sequence analysis were produced by digestion of the enzyme with cyanogen bromide or *Staphylococcus aureus* V8 protease. Additional fragments were generated by tryptic digestion of the protein after derivatisation of the lysine residues with maleic anhydride. In all cases, the peptides which resulted from these digests were purified by reverse-phase HPLC, using a RP 300 column (25 × 4.6 mm) (Brownlee) and a solvent gradient of 10–60% acetonitrile in water with 0.06% trifluoroacetic acid in each solvent. Sequence analysis was performed by automated Edman degradation using an Applied biosystems 470A gas phase protein sequencer (Kelvin Close, Warrington, U.K.).

Elemental analysis. A sample of the protein was analysed for the presence of a metal cofactor by inductively coupled plasma spectrophotometry (ICP) by Butterworth Laboratories Ltd (Teddington, U.K.).

RESULTS

Properties of a Walker 256 cell protein with a nitroreductase activity

A pure protein with a molecular weight of 33.5 kDa (as determined by SDS–polyacrylamide electrophoresis and by gel filtration chromatography) which catalyses the reduction of CB 1954 (5-aziridin-1-yl)-2,4-dinitrobenzamide) to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide has been isolated from Walker 256 tumour cells [3]. The protein has a distinctive absorption at 450 nm (Fig. 1) which gives it a characteristic yellow colour and comparison of the UV-visible absorption spectrum with those of some flavoproteins suggested the presence of a flavin coenzyme. By heating the enzyme at 56° for 20 min this flavin group could be separated from the protein (indicating that the flavin

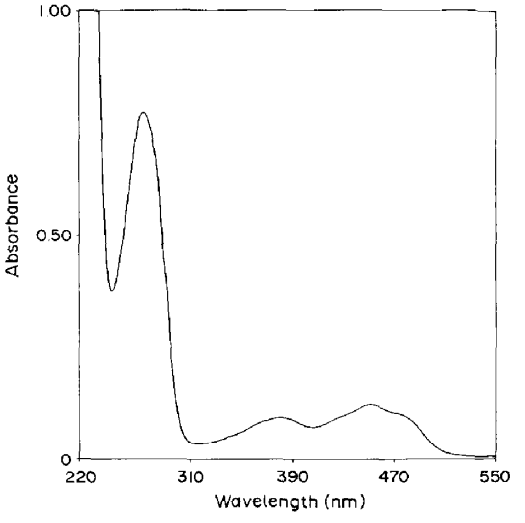

Fig. 1. The UV-visible absorption spectrum of the nitroreductase enzyme isolated from Walker cells. The spectrum was recorded in a 10 mm pathlength. All measurements were made in 0.1 M sodium phosphate buffer, pH 7 and measurements were corrected for the absorption of the solvent. Protein concentration was 319 μg/ml. Peaks of absorption were found at 272 nm ($E = 6.65 \times 10^4$), 380 nm ($E = 8.1 \times 10^3$) and 451 nm ($E = 1.06 \times 10^4$).

is tightly, but not covalently bound) and isolated by ultrafiltration. The UV-visible spectrum of this filtrate suggested that this prosthetic group was FAD as opposed to FMN, the ratios of UV absorption in 0.1 M sodium phosphate buffer (pH 7) at 375 nm to 450 nm and at 260 nm to 450 nm being 0.79 and 3.6 respectively. These values are much closer to those published for FAD than those for FMN [8]. The coenzyme was confirmed to be FAD by HPLC using the method of Pietta *et al.* [9].

From the absorption spectrum of the protein (Fig. 1), the concentration of FAD in this 390 μg/ml solution was calculated to be 10.9 μM (the molar extinction coefficient of FAD is 11,300 [8]). For a protein a molecular weight of 33.5 kDa, the protein concentration would be 11.64 μM. Thus there is a single FAD group per protein molecule. Molar extinction coefficients of the protein, in 0.1 M sodium phosphate buffer (pH 7), were calculated to be 6.65×10^4 and 1.06×10^4 at 272 nm and 451 nm respectively. a 1 mg/ml solution of the protein would give absorbances (10 mm pathlength) of 2.1 and 0.34 at 270 nm and 450 nm respectively.

Analysis of the protein by ICP showed that the protein contained no typical metal cofactor atoms such as iron, zinc, molybdenum, copper or manganese.

The relative rates of reduction of some other nitrobenzamides and nitrobenzenes by the Walker enzyme are shown in Table 1. Although the products of these reactions were not characterised it is apparent that the enzyme is not a general nitroreductase and as indicated by this limited range of substrates the reductase activity appears to be confined to certain dinitrobenzamides or amino-nitrobenzamides. However, the very rapid rate of reduction of 2,6-dichlorophenolindophenol (DCPIP) would suggest that this enzyme is able to catalyse electron transfer reactions other than to nitro groups.

For the reduction of CB 1954 both NADH and NADPH could be used with about equal efficiency as electron donors and this reaction proceeded under either air or nitrogen at equal rates. The stoichiometry of the reaction, indicated from 12 determinations, was 2–3 moles of NADH oxidised per mole of CB 1954 reduced (data not shown).

Identification of the protein with the nitroreductase activity

The purified protein was subject to amino acid sequence analysis but it gave no N-terminal residue. However, digestion of the protein with either cyanogen bromide, *Staphylococcus aureus* V8 protease or, after derivatisation with maleic anhydride, with trypsin, generated a series of peptides which gave sequence at about 80% of the N-terminal yield in each case. In this way, a series of overlapping sequences was generated which gave four sequences of over forty amino acid residues each. These partial protein sequences showed an all but complete homology with the amino acid sequence deduced from the cloned mRNA from the gene coding for NAD(P)H dehydrogenase (quinone) in rat liver [7, 10, 11] (Table 2). Positions in these sequences which gave no clear residues corresponded with either tryptophan or cysteine residues in the deduced sequence, amino acids which are readily degraded during sequence analysis. In addition, several short sequences were also obtained which could be assigned to the deduced sequence and no sequences were found which did not agree with the deduced sequence. A search of a protein sequence data base [12] showed no match or homology with any other sequenced protein and it was concluded that the nitroreductase protein isolated from Walker 256 cells was a form of NAD(P)H dehydrogenase (quinone).

The vitamin K_2 analogue, menadione, has been used as a substrate in assays for NAD(P)H dehydrogenase (quinone) [7]. The Walker cell nitroreductase enzyme gave a K_m value of 1.33 μM with respect to menadione, in the presence of excess NADH (500 μM) at 37° (data not shown). The specific activity of the enzyme, as isolated from Walker cells, was measured at 37° with a menadione concentration of 10 μM, NADH at 500 μM and the final electron acceptor, cytochrome *c* at 70 μM. It was calculated to be 1.94×10^9 units (nmol cytochrome *c* reduced per min) per mg of protein.

Table 1. The relative rates of reaction of various nitrobenzamides and nitrobenzenes with the Walker cell nitroreductase enzyme

Substrate	Relative rate of reaction (%)
CB 1954	100
CB 1954 + nitrogen	84
CB 1954 + NADPH	132
2,4-Dinitro-5-(2'-hydroxyethylamino)benzamide	<1.0
2-Amino-5-(aziridin-1-yl)-4-nitrobenzamide	412
4-Amino-5-(aziridin-1-yl)-2-nitrobenzamide	26
5-Chloro-2,4-dinitrobenzamide	74
3,5-Dinitrobenzamide	37
2-Nitrobenzamide	<1.0*
3-Nitrobenzamide	<1.0*
4-Nitrobenzamide	<2.0*
2.4-Dinitrophenol	<1.0*
DCPIP	>10,000

Reaction rates were determined either directly by loss of the substrate or by the resulting oxidation of NADH(*). All reactions were carried out at 37° in air, with NADH as electron donor, unless otherwise indicated.

Table 2. A comparison of the complete sequence of NAD(P)H dehydrogenase (quinone) [7] and the partial protein sequences obtained from the Walker nitroreductase enzyme (bold print)

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                    10                  20                  30
M A V R R A L I E L A H A E R T S F N Y A M K E A A V E A L K K K
                                      E A A (V) E A L K K

            40                  50                  60
G W E V V E S D L Y A M N F N P L I S R N D I T G E P K D S E N F
G — E V — E — D L Y A M N F N P L I S R N D I T G E P K D S E N F

        70                  80                  90
Q Y P V E S S L A Y K E G R L S P D I V A E Q K K L E A A D L V I
Q Y P V E S S L A Y K E G R L — P D I V A E Q K K L E A A D L V I

100                 110                 120                 130
F Q F P L Y W F G V P A I L K G W F E R V L V A G F A Y T Y A T M
F Q F P L Y — F G V P A I L K G — F E R V L (V) A G F A Y T Y A T

            140                 150                 160
Y D K G P F Q N K K T L L S I T T G G S G S M Y S L Q G V H G D M
                                          Y — L Q G V H G D M

        170                 180                 190
N V I L W P I Q S G I L R F C G F Q V L E P Q L V Y S I G H T P P
N V I L — P I Q S G I L R F (C) G F Q V L E P Q L V Y S I G H T P P

    200                 210                 220                 230
D A R V Q V L E G W K K R L E T V W E E S P L Y F A P S S L F D L
D A R V Q V L E G - K (K) R L E T V E E E S P L Y F A P S (E) L F D L

                240                 250                 260
N F Q A G F L L K K E V Q E E Q K K N K F G L S V G H H L G K S I
N F Q A G F L (L) — — — — Q E E Q K K N K F G L S V G H H L G K I (I)

            270
P A D N Q I K A R K
P A D N Q I
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A "—" indicates that no amino acid was distinguished above background during sequence analysis and "( )" indicates that the identification of the residue was not certain.

Inhibitors of the Walker cell enzyme

The enzyme NAD(P)H dehydrogenase (quinone) is characterised by the ability of dicoumarol to inhibit the above reduction of menadione. A K_i value for dicoumarol of approximately 10 nM was obtained by measuring the effect of this compound on the rate of reduction of menadione, by the nitroreductase enzyme isolated from Walker cells, with varying NADH concentrations (0.1–0.5 mM). The extent of inhibition by dicoumarol and other compounds of the "menadione reductase" activity of the Walker nitroreductase enzyme, under standard conditions are shown in Table 3. As judged by the low concentrations required to reduce enzyme activity to either 50% or 10% of the control rates, dicoumarol is a potent inhibitor of the enzyme (>90% inhibition at concentrations greater than 0.2 μM). The other compounds inhibited the activity of the enzyme in the millimolar concentration range. Both 2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide and 5(4)-aminoimidazole-4(5)-carboxamide inhibited the enzyme to similar extents. As expected from a known substrate, CB 1954 also inhibited the enzymic reduction of menadione. That the actual mechanisms by which these compounds inhibit the enzyme might be complex was indicated by the fact that only dicoumarol and warfarin gave the predicted linear result when the reciprocal of the rate of reaction was plotted against the inhibitor concentration (data not shown).

Table 3. The ability of various compounds to inhibit the reduction of menadione by the Walker nitroreductase enzyme

Compound	Concentration (mM) to reduce the reaction rate to 50%	10%
Dicoumarol	0.015×10^{-3}	0.16×10^{-3}
Warfarin	0.03	0.31
5(4)-Aminoimidazole-4(5)-carboxamide	1.39	1.91
2-Phenyl-5(4)-aminoimidazole-4(5)-carboxamide	1.19	2.25
CB 1954	0.50	0.97

DISCUSSION

The Walker tumour is a hydrocarbon-induced rat mammary carcinoma characterised by its exceptional sensitivity to CB 1954 [3]. As we have previously shown, activation of the monofunctional alkylating agent CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) occurs by reduction of the 4-nitro group to the hydroxylamine derivative, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a difunctional compound capable of forming DNA interstrand crosslinks [1, 2]. The formation of this compound in Walker cells accounts for the highly specific cytotoxic action of CB 1954 towards these cells.

This activation of CB 1954 occurs by the action of a specific nitroreductase enzyme which we have isolated from Walker cells. The enzyme, as isolated, is a monomeric FAD-containing flavoprotein with a molecular weight of 33.5 kDa which can use either NADH or NADPH to catalyse the reduction of CB 1954 to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide. These reductions require 4 and 6 electrons respectively, and this seems to be reasonably well reflected in the reaction stoichiometry with 2–3 moles of NADH (a 2-electron donor) being oxidised per mole CB 1954 reduced. Reduction of CB 1954 takes place at equal rates in air or nitrogen indicating that it is not inhibited by oxygen and suggesting that reduction takes place by the initial addition of at least two-electrons to avoid the redox cycling of the one-electron radical anion ($R\text{-}NO_2^{\cdot-}$) with oxygen which would reform the parent compound [13]. Enzymes such as NADPH cytochrome P450 reductase and xanthine oxidase, which can only transfer a single electron at a time, are incapable of reducing nitro groups in air.

The rates of reduction of other nitro compounds by the enzyme as shown in Table 1, indicate that CB 1954 appears to be one of the most readily reduced substrates. On the other hand some closely-related analogues are not reduced by the enzyme at all. For example, 2,4-dinitro-5-(2′-hydroxyethylamino)-benzamide is not reduced by the enzyme although it differs from CB 1954 only in that the cyclic aziridine ring has been opened by hydrolysis. Ring-opening occurs during the reaction of CB 1954 with nucleophilic sites, as when it becomes monofunctionally-bound to DNA via its 2′ carbon atom. This result would suggest that such monofunctionally-bound CB 1954 could not be further activated to a form able to cross-link DNA. Hence, the initial reactions leading to the formation of DNA crosslinks, would seem to be via the hydroxylamine moiety. However, there is no strict requirement for the presence of the aziridine group in order for reduction to occur. For instance, both 5-chloro-2,4-dinitrobenzamide and 3,5-dinitrobenzamide are reduced by the enzyme. The effect of the carboxamide group on the ability of the enzyme to reduce a compound is unclear from studies with the selection of compounds used in this study. On the other hand, structure activity studies carried out on a large number of CB 1954 analogues suggested that the presence of the carboxamide group improved the therapeutic ratio of such compounds toward the Walker tumour *in vivo* [14, 15]. This may be related to their relative abilities to be reduced by the enzyme. Such a possibility would explain why 1-(aziridin-1-yl)-2,4-dinitrobenzene (CB 1837) still possesses a specific action against the Walker tumour but is much less active than CB 1954 [16].

The purified protein was subject to sequence analysis. The results shown in Table 2 indicate the almost complete homology of our sequenced fragments with the amino acid sequence deduced from the cloned mRNA of the gene coding for the FAD-containing flavoenzyme NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2) as isolated from rat liver. The complete sequence was first deduced by Robertson *et al.* [7]. Bayney *et al.* [10] subsequently reported a different sequence around the NH_2-terminus. However, Bayney and Pickett [11] later ascribed this to a sequencing artefact and confirmed the original sequence reported by Robertson *et al.* [7]. NAD(P)H dehydrogenase (quinone) shows no amino acid homology with other flavoenzymes, suggesting it forms part of a new gene family which evolved independently from those genes encoding other flavoproteins [7]. The enzyme was originally described by Ernster and Navazio [17] who named the enzyme DT diaphorase in deference to its unique ability to utilise both NADH and NADPH as electron donors (at this time these were abbreviated as *D*PNH and *T*PNH respectively). It catalyses the 2-electron reduction of quinones to hydroquinones, reactions highly inhibited by dicoumarol and other vitamin K antagonists (for a review see [18]).

That the Walker nitroreductase enzyme was a form of NAD(P)H dehydrogenase (quinone) was further confirmed by its ability to reduce menadione in the presence of NADH or NADPH and by the fact that this reaction was strongly inhibited by dicoumarol and warfarin, both known inhibitors of NAD(P)H dehydrogenase (quinone). Dicoumarol has been described as a specific inhibitor of NAD(P)H dehydrogenase (quinone) and is one of the most potent enzyme inhibitors known [18–20], values for the inhibition constants (K_i) values, with respect to either NADH or NADPH, of between 50 pM and 200 nM having been reported [20, 21]. The K_i value of 10 nM for dicoumarol with respect to NADH obtained with the Walker enzyme is well within this range. Dicoumarol also inhibited the reduction of CB 1954 by the Walker enzyme (data not shown). This enzyme was inhibited by both 5(4)-aminoimidazole-4(5)-carboxamide and 2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide which have not previously been reported to be inhibitors of NAD(P)H dehydrogenase (quinone). However, these compounds diminish the antitumour effects of CB 1954 in the Walker tumour *in vivo*, and such observations led to the theory that CB 1954 acted as an antimetabolite [4]. The fact that these compounds have now been shown to be able to inhibit the enzyme that activates CB 1954 could explain this *in vivo* antagonistic effect. While 2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide was shown to be more potent in this respect, both compounds inhibited the isolated Walker enzyme to a similar extent. This difference in their *in vivo*, as opposed to their *in vitro* effects may be due to differences in their pharmacological properties. It would be predicted from

these results that dicoumarol would be a very potent protective agent against CB 1954-induced toxicity to the Walker cell both *in vivo* and *in vitro*.

NAD(P)H dehydrogenase (quinone) has been reported to exist both as a dimer and as a monomer. Huang *et al.* [22] described the enzyme as a dimer, with an apparent molecular weight of 54 kDa, consisting of identical subunits each of which contained a single FAD. From the amino acid sequence, the (subunit) molecular weight was calculated to be 30,946, which compared well with the size of the rat enzyme (32 kDa) [7] and the Walker enzyme (33.5 kDa) as estimated by SDS-polyacrylamide gel electrophoresis. By gel filtration chromatography the molecular weight of the Walker cell enzyme was estimated to be about 35 kDa which indicates that the enzyme, as isolated from Walker cells, is a functional monomer. The spectral data indicated a single FAD per protein molecule.

NAD(P)H dehydrogenase (quinone) is known to be widely distributed throughout eukaryotes being most abundant in mammalian liver. It is known to exist in various forms, two quite different forms having been isolated from murine liver [21] while up to three forms may be present in rat liver cytosol [7] whilst another is thought to be membrane bound [18]. In the rat there appears to be only a single gene which codes for this protein, with a size of about 14–16 kilobases [7], which raises some questions on the genomic basis of this diversity. The enzyme has been well-studied due to its role in the detoxification of quinones. Such compounds are thought to exert some of their toxicity following their one-electron reduction [by enzymes other than NAD(P)H dehydrogenase (quinone)] to semiquinone free radicals. These radicals can then react with oxygen to regenerate the quinone (which can then be re-reduced) and produce superoxide (O_2^-) and other reactive oxygen species. NAD(P)H dehydrogenase (quinone) can circumvent this oxygen stress of quinone cycling by a 2-electron reduction of the compound to a hydroquinone which can be conjugated to glucuronic acid [18]. NAD(P)H dehydrogenase (quinone) is inducible by polycyclic hydrocarbons and in the mouse this is known to be governed by the aromatic hydrocarbon-responsive (Ah) locus [23]. Elevated levels have been reported in tumours [24, 25] and its levels have been reported to be so high in the neoplastic foci of chemically-induced hepatocarcinoma that they can be used to image them [26].

Our observation that NAD(P)H dehydrogenase (quinone) as isolated from Walker cells, can activate CB 1954 by a 4-electron reduction to the hydroxylamino derivative is of interest for a number of reasons.

Firstly, an inducible enzyme which is involved in drug detoxification has been shown to be also capable of activating another compound to a cytotoxic form.

Secondly, nitro groups are not generally considered to be substrates for NAD(P)H dehydrogenase (quinone). Although NAD(P)H dehydrogenase (quinone) has been suggested to be involved in the reduction of nitroimidazoles [27] this has never been demonstrated. Sugimura *et al.* [28] identified NAD(P)H dehydrogenase (quinone) as the enzyme which activates 4-nitroquinoline-*N*-oxide (again by reduction of a nitro group to a hydroxylamino), primarily based on dicoumarol inhibition studies. However, most cell lines in culture show very similar sensitivities to 4-nitroquinoline-*N*-oxide in contrast to the expected variation in the levels of NAD(P)H dehydrogenase (quinone). Moreover, induction of NAD(P)H dehydrogenase (quinone) does not seem to potentiate 4-nitroquinoline-*N*-oxide toxicity [29]. These ambiguous results may be explained by the recent evidence that dicoumarol inhibits other enzyme systems and should therefore no longer be considered a specific inhibitor for NAD(P)H dehydrogenase (quinone) [30].

Thirdly, the activation of CB 1954 has only been described in Walker cells. However, sensitivity to CB 1954 can be generated in resistant cells solely by the presence of the Walker enzyme in the culture medium [2]. NAD(P)H dehydrogenase (quinone) is known to be a ubiquitous enzyme and it might therefore be expected that a much larger number of cell types would be sensitive to CB 1954. That this does not seem to be the case may be because NAD(P)H dehydrogenase (quinone) exists in various forms and it is possible that either the Walker form is unique in its nitroreductase ability or that coenzyme levels in Walker cells are particularly high. Alternatively, the ability to reduce nitro compounds may be a property only of the rat NAD(P)H dehydrogenase (quinone) or one of its isoenzymes (the levels of which may not correlate with the measured NAD(P)H dehydrogenase (quinone activity). This could explain why CB 1954 is less toxic to mice than to rats [31], and indeed preliminary results indicate that some other rat tumour cells are sensitive to CB 1954, although the basis of their sensitivity is yet to be determined. Finally, high levels of the enzyme may be required to reduce CB 1954 and the required levels are only found in cells such as the Walker. However, contrary to this last possibility, quite small amounts of Walker enzyme have been shown to activate CB 1954 *in vitro* and kill normally resistant cells [3]. Further studies will be directed towards establishing the relationship between CB 1954 cytotoxicity and NAD(P)H dehydrogenase (quinone) levels in various cell lines. It also remains to be determined whether human NAD(P)H dehydrogenase (quinone) can reduce CB 1954.

In summary, we have shown that CB 1954 can be activated to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a difunctional alkylating agent by a previously undescribed nitroreductase activity of NAD(P)H dehydrogenase (quinone) isolated from Walker tumour cells. The selective bio-activation of CB 1954 could lead to the development of new anti-cancer strategies. Antibodies to the enzyme could lead to the detection of CB 1954-sensitive tumours or the enzyme itself could be targeted to specific tumours by coupling it to a tumour specific antibody [32]. Thus CB 1954 could be seen as a pro-drug for enzyme-mediated chemotherapy.

Acknowledgements—This work was supported by a joint grant from the Medical Research Council and the Cancer Research Campaign. We wish to record our appreciation to Prof. B. Ketterer (CRC Molecular Toxicology Research

Group, Middlesex Hospital Medical School) for helpful advice on protein sequencing and to Dr. S. Neidle (CRC Biomolecular Structure Unit, ICR) for access to a protein sequence data base.

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