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THE BIOACTIVATION OF 5-(AZIRIDIN-1-YL)-2,4-DINITROBENZAMIDE (CB1954)—II

A COMPARISON OF AN *ESCHERICHIA COLI* NITROREDUCTASE AND WALKER DT DIAPHORASE

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Abstract—A nitroreductase enzyme that has been isolated from *Escherichia coli* B is capable of bioactivating CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] to a cytotoxic agent, a property shared with the mammalian enzyme Walker DT diaphorase [NAD(P)H dehydrogenase (quinone), EC 1.6.99.2] as isolated from Walker cells. In contrast to Walker DT diaphorase, which can only reduce the 4-nitro group of CB1954, the *E. coli* nitroreductase can reduce either (but not both) nitro groups of CB1954 to the corresponding hydroxylamino species. The two hydroxylamino species are formed in equal proportions and at the same rates. CB1954 is reduced much more rapidly by the *E. coli* nitroreductase than by Walker DT diaphorase. If the reduction of CB1954 was carried out in the presence of V79 cells (which are insensitive to CB1954) a large cytotoxic effect was evident. This cytotoxicity was only observed under conditions in which the *E. coli* nitroreductase or Walker DT diaphorase reduced the drug. It is proposed that *E. coli* B nitroreductase would be a suitable enzyme for antibody-directed enzyme prodrug therapy (ADEPT) in combination with CB1954.

CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide§] was originally studied because it exhibited a highly specific toxicity towards the Walker 256 rat tumour when grown either in the rat [1] or in cell culture [2, 3]. Selective toxicity was not expected from a monofunctional alkylating agent such as CB1954. The basis of this selective action is the formation of DNA interstrand crosslinks in toxically affected cells, but not in non-CB1954-sensitive cells [3]. Thus, in Walker cells, CB1954 is acting as a difunctional, not a monofunctional, alkylating agent. Crosslink formation is a result of the bioactivation of the drug, by the specific reduction of the 4-nitro group, to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide [4]. The enzyme that performs this aerobic reduction of CB1954 in Walker cells has been isolated, purified and identified as a form of DT diaphorase [NAD(P)H dehydrogenase (quinone), EC 1.6.99.2] [5], an enzyme involved in the metabolism of a number of other chemotherapeutic agents [6]. Other rat tumour cell lines are also sensitive to CB1954, as predicted by their levels of Walker DT diaphorase [7]. The 4-hydroxylamino reduction product is cytotoxic even towards those cells that are resistant to CB1954 and forms crosslinks in their DNA [4]. This is not a direct reaction and the 4-hydroxylamine itself undergoes a second activation step by a non-enzymatic reaction with a thioester (such as acetyl CoA) to form the final DNA reactive species which is probably 4-(*N*-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide [8]. The increase in cytotoxicity upon bioactivation of CB1954 is dramatic and can be up to a 100,000-fold on a dose basis [3]. This increase in cytotoxicity, which is greater than that predicted for the conversion of a mono- to difunctional alkylating agent, is probably a consequence of the very high frequency of crosslinks formed and their poor repair [9].

Unfortunately, the human form of Walker DT diaphorase is intrinsically less able to perform this bioactivation and thus human tumour cells remain relatively insensitive to CB1954 [7]. To overcome this problem it has been proposed that rat DT diaphorase could be targeted to a human tumour by conjugating it to an antibody [4, 10]. This concept has been termed ADEPT (antibody-directed enzyme prodrug therapy) [11, 12]. In ADEPT endogenous enzymes are not being exploited and, indeed, it is fundamental to the concept that the prodrug (such as CB1954) is not activated by enzymes in normal human tissues. The biochemical properties of enzymes from mammalian species may not be expected to be sufficiently diverse, and although we have demonstrated an exploitable difference between the human and rat forms of DT diaphorase, candidate enzymes for ADEPT have tended to be bacterial in origin. We have recently purified a nitroreductase enzyme from *E. coli* B that is also capable of reducing CB1954 [13]. In view of our interest in using CB1954 in ADEPT we now report on the

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§ Abbreviations; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; ADEPT, antibody-directed enzyme prodrug therapy.

ability of this bacterial enzyme to bioactivate CB1954 to a cytotoxic species.

MATERIALS AND METHODS

Materials. All chemicals and reagents were supplied by the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. CB1954 and its derivatives were synthesized or supplied by Prof. M. Jarman and Dr D. Wilman, Institute of Cancer Research, Sutton, U.K. [^{3}H]CB1954 was prepared by Amersham International (Amersham, U.K.) [8].

E. coli B nitroreductase and DT diaphorase from Walker 256 cells were purified as described previously [4, 13].

Enzymatic reduction of CB1954. CB1954 (100 μM with [U-^{3}H]CB1954 added to give an activity of 1.6×10^5 dpm/nmol) and NADH or NADPH (500 μM) were incubated with enzyme (2 μg/mL *E. coli* nitroreductase or 35 μg/mL Walker DT diaphorase) in 100 mM sodium phosphate buffer (pH 7) under either air or helium [4]. At various times aliquots (10 μL) were injected onto a Partisphere SCX (110 × 4.7 mm) HPLC column and eluted isocratically (2 mL/min) with 100 mM NaH_2PO_4. The eluate was continuously monitored for absorption at 260, 310 and 340 nm using a diode-array detector and the spectra of eluting components recorded. Samples (0.5 mL) were collected and their tritium activity determined by liquid scintillation counting. This separation system could resolve all the expected reduction products as shown in Fig. 2. To confirm further the identity of any reduction products, they were also compared with chemically synthesized standards on an alternative separation system. The reduction mixture was injected on to an ODS-5 reverse phase HPLC column and eluted (1 mL/min) with a methanol gradient (0–30% linear over 30 min, 30–100% linear over 10 min) in 0.01 M sodium phosphate buffer (pH 7).

Determination of effects of agents on cell survival. To determine the effect of enzyme-activated CB1954 on the survival of V79 cells, 1 mL volumes of V79 cells in tissue culture medium (2×10^5/mL) were incubated with 50 μM CB1954, 500 μM NADH, and 2 μg/mL *E. coli* nitroreductase or 50 μg/mL Walker DT diaphorase. After a 2 hr incubation at 37°, the cells were harvested and assayed for their colony-forming ability, and the supernatant assayed for the concentration of remaining CB1954 by HPLC as described previously [4].

RESULTS

The products of the enzymatic reduction of CB1954

Walker DT diaphorase yielded a single product and this was shown to be 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (Figs 1 and 2), in agreement with previously published data [7]. Reduction of CB1954 by the *E. coli* nitroreductase resulted in the formation of two products (Figs 1 and 2). By comparison of retention times and spectral characteristics with known standards, these products were shown to be 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 5-(aziridin-1-yl-2-hydroxylamino-4-nitrobenzamide. No other CB1954 metabolites were found. This was also confirmed by the distribution of radiolabelled CB1954 using the alternative reverse-phase separation system. Tritium activity was only found in peaks corresponding to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 5-(aziridin-1-yl-2-hydroxylamino-4-nitrobenzamide. 5-(Aziridin-1-yl)-2-hydroxylamino-2-nitrobenzamide is less stable than the 4-hydroxylamino derivative and, at later times, some tritium activity was also found in peaks corres-

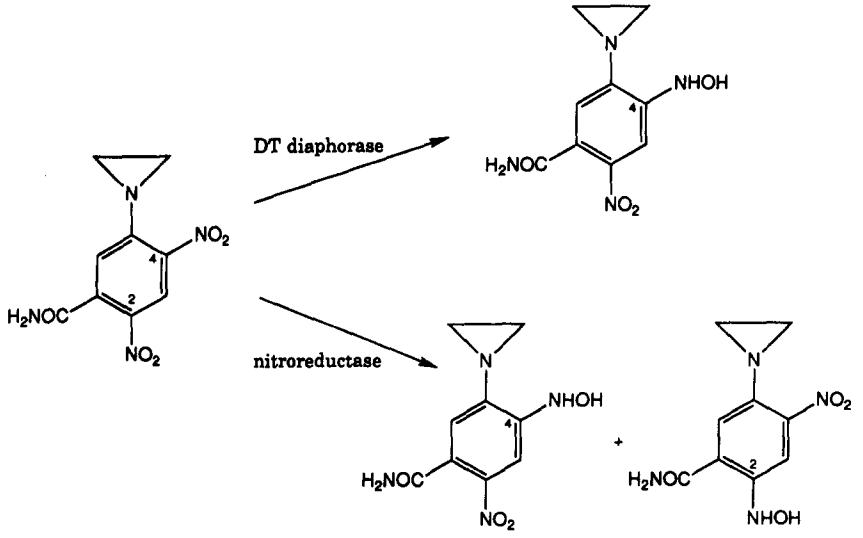

Fig. 1. The formula of CB1954 and the products formed by its reduction by either Walker DT diaphorase or *E. coli* nitroreductase. DT diaphorase only generates the 4-hydroxylamino form whereas the nitroreductase generates both the 2- and 4-hydroxylamino forms in equal proportions.

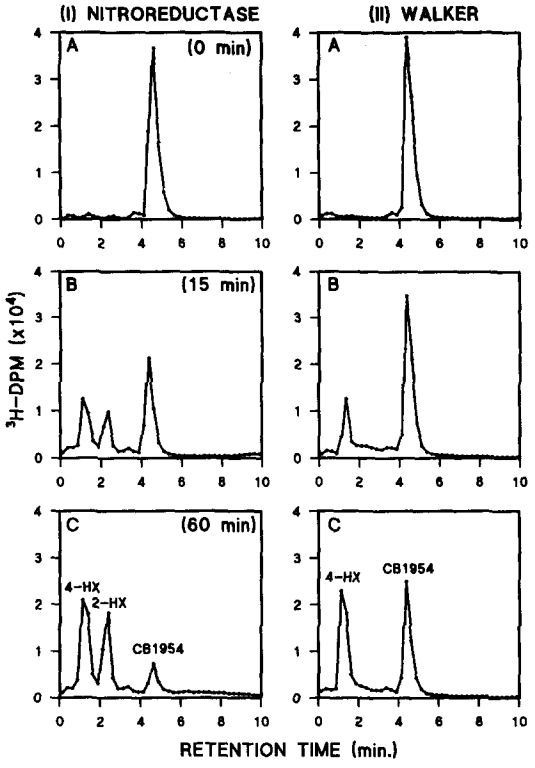

Fig. 2. The products generated by the aerobic reduction of CB1954 by (I) 2 μg/mL *E. coli* nitroreductase for (A) 0 min; (B) 15 min; (C) 60 min or (II) by 35 μg/mL Walker DT diaphorase for the same times. Reduction conditions are as described in the text, the initial concentrations of CB1954 and NADH were 100 and 500 μM, respectively. Elution times of the standards were: CB1954, 4.4 min; 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (2-HX), 2.4 min; 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (4-HX), 1.3 min; 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide, 6.1 min and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide, 3.1 min.

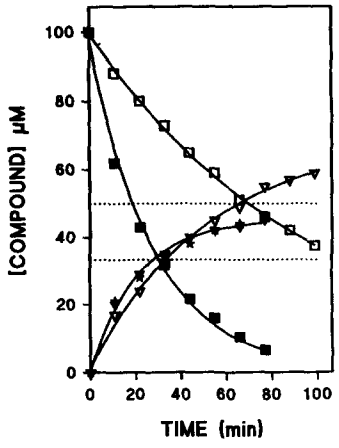

Fig. 3. The aerobic reduction of CB1954 (squares) and the resulting formation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (triangles) and 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (★) by either the *E. coli* nitroreductase (solid symbols) or Walker DT diaphorase (open symbols). The reduction conditions are as described in the text and NADH (500 μM) was used as a cofactor. Enzyme concentrations were 2 and 35 μg/mL for the *E. coli* and Walker enzymes, respectively. 5-(Aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide was not formed by Walker DT diaphorase.

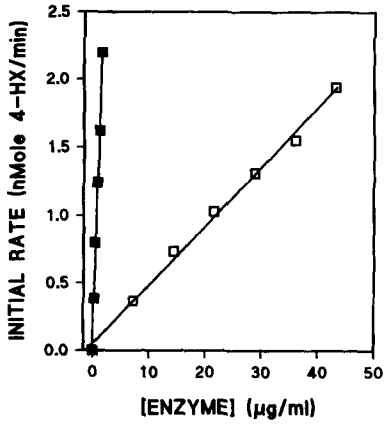

Fig. 4. The effect of the concentration of *E. coli* nitroreductase (■) or Walker DT diaphorase (□) on the initial rate of formation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (4-HX) from CB1954. Reduction conditions are as detailed in the text, the initial concentrations of CB1954 and NADH were 100 and 500 μM, respectively. Linear regression analysis gave initial rates of 1.31 nmol/min/μg *E. coli* nitroreductase and 0.042 nmol/min/μg of the Walker enzyme.

ponding to decomposition products of the 2-hydroxylamine (data not shown). From the proportions of radioactivity in the appropriate peaks the ratio of the 2 and 4-hydroxylamine derivatives was estimated to be 1:1. This was confirmed when the peak areas of the appropriate products were calibrated against the known standards (Fig. 3). In further confirmation of the equal formation of both the 2- and the 4-hydroxylamines by the nitroreductase, 33 μmol of 4-hydroxylamine was formed when 67 μmol of CB1954 was reduced by the nitroreductase. In contrast, 50 μmol of 4-hydroxylamine was formed by the reduction of 50 μmol of CB1954 by the Walker DT diaphorase (Fig. 3). On the basis of initial rates of 4-hydroxylamine formation the rate of CB1954 reduction is directly proportional to the concentration of either enzyme (Fig. 4). Under the standard conditions used the *E. coli* nitroreductase was 31-fold more active per μg protein than was Walker DT diaphorase at producing the 4-hydroxylamine (or 62-fold more active by CB1954 reduction) (Fig. 4).

The rate of reduction of CB1954 and spectrum of product formation by *E. coli* nitroreductase was the same when the cofactor was either NADPH or NADH and when the reduction was performed under helium or in air (data not shown). A similar

Table 1. The effect of CB1954 on the survival of V79 cells in the presence of the *E. coli* nitroreductase or Walker cell DT diaphorase

Treatment	% Survival	% Drug reduction
Control	100	—
+ 500 μM NADH	100	—
+ 50 μM CB1954	100	<1.0
+ NADH + CB1954	41	<1.0
+ *E. coli* nitroreductase (NR) (2 μg/mL)	94	—
+ NR + 50 μM CB1954	99	<1.0
+ NR + 50 μM CB1954 + 500 μM NADH	0.024	72
+ Walker DT diaphorase (50 μg/mL)* + 50 μM CB1954 + 500 μM NADH	<0.01	51

All treatments were for 2 hr at 37° and the cells were then plated out for their resulting colony-forming ability. NADH was used as a cofactor for both enzymes.
* Data from Knox *et al.* [5].

result has previously been reported for Walker DT diaphorase [4].

The cytotoxicity produced by the enzymatic reduction of CB1954

In order to demonstrate that the nitroreductase was producing a cytotoxic species, the reduction of CB1954 was carried out in the presence of V79 cells (which are insensitive to CB1954). As shown in Table 1, a dramatic cytotoxic effect was observed in V79 cells—but only under those conditions in which the nitroreductase reduced CB1954. Over the 2 hr treatment period 2 μg *E. coli* nitroreductase reduced about 35 nmol of CB1954 and cell survival was reduced to 0.024% of control. In the presence of the Walker DT diaphorase cell survival was reduced to less than 0.01%. However, 50 μg of Walker DT diaphorase was required to reduce about 25 nmol of CB1954.

DISCUSSION

We have shown that an isolated nitroreductase enzyme from *E. coli* can, like Walker DT diaphorase, reduce CB1954 to the cytotoxic 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide derivative. The purified *E. coli* nitroreductase enzyme is a monomeric FMN-containing flavoprotein with a molecular mass of 24 kDa [13], whereas Walker DT diaphorase contains FAD and has a molecular mass of about 33 kDa [5]. There is no sequence homology between the two enzymes [11], however both enzymes can reduce CB1954 in air using either NADH or NADPH as a cofactor. They also both reduce quinones and are inhibited by dicoumarol [11]. However, there would appear to be distinct differences in their mechanism of reduction of CB1954. While Walker DT diaphorase reduces CB1954 to only the 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide derivative, reduction of CB1954 by the nitroreductase results in equimolar amounts of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide. Thus, in contrast to Walker DT diaphorase, *E. coli* nitroreductase can reduce either the 2- or 4-nitro groups of CB1954 with equal rates. However, no products are formed in which both nitro groups have been reduced, thus, once one nitro group has been reduced, the *E. coli* nitroreductase cannot then reduce the other nitro group. Although they are not products of the reduction of CB1954 by Walker DT diaphorase, both the 2-hydroxylamine and 2-amino derivatives of CB1954 are substrates for the Walker enzyme [5]. 5 - (Aziridin - 1 - yl) - 4 - hydroxylamino - 2 - nitro - benzamide is a potent cytotoxic agent capable of producing DNA–DNA interstrand crosslinks in cells. In contrast, the 2-hydroxylamino species is much less cytotoxic and cannot produce interstrand crosslinks [4]. It is, nevertheless, much more cytotoxic than the parent compound, CB1954 [4].

The activity of the *E. coli* nitroreductase towards CB1954 is also much higher. Under our standard (but not saturating) conditions the *E. coli* nitroreductase reduces CB1954 about 60-fold faster (and produces the 4-hydroxylamine 30-fold faster) than Walker DT diaphorase on a weight of enzyme basis. This agrees well with the measured kinetic parameters of the enzymes for CB1954 (*E. coli* nitroreductase k_{cat} = 360 min^{-1} [13], Walker DT diaphorase k_{cat} = 4.1 min^{-1} [7]. Furthermore, as the K_m of nitroreductase for NADH or NADPH is much lower than that of Walker DT diaphorase [5, 12], the nitroreductase would be expected to reach its limiting velocity at significantly lower concentrations of cofactor. However, we have recently shown that the measured K_m of Walker DT diaphorase for its cofactors depends upon the actual assay used [13]. Although the K_m of Walker DT diaphorase for NAD(P)H is about 75 μM when reducing the quinone compound menadione [5], it approaches micromolar levels (below limits of detection) when CB1954 is reduced [14].

Chinese hamster V79 cells are normally insensitive to CB1954 because they have very low levels of DT diaphorase [9]. The *E. coli* nitroreductase was capable of activating CB1954 extracellularly in the presence of NADH to a form cytotoxic to these cells. This is fully consistent with the formation of 5 - (aziridin - 1 - yl) - 4 - hydroxylamino - 2 - nitro -

benzamide. Compared to Walker DT diaphorase much less enzyme was required to produce a potent cytotoxic effect in V79 cells, again this is consistent with the more rapid reduction of CB1954 by the *E. coli* nitroreductase. However, it was noted that Walker DT diaphorase appeared to cause more cytotoxicity, whilst reducing less CB1954 than *E. coli* nitroreductase. This would be expected because only half the CB1954 will be reduced by the bacterial enzyme to form the highly cytotoxic 4-hydroxylamine; the remainder will produce the much less cytotoxic 2-hydroxylamine form. In contrast, all the CB1954 reduced by Walker DT diaphorase will form the highly cytotoxic species.

In conclusion, the nitroreductase enzyme that we have isolated from *E. coli* can, in common with DT diaphorase isolated from mammalian Walker cells, reduce the prodrug, CB1954 to its cytotoxic form. Compared to the Walker cell enzyme the bacterial nitroreductase can perform the bioactivation much more rapidly. It is possible that the bacterial enzyme may have a role in the therapy of human tumours, whereby a conjugate made between the nitroreductase and a tumour-specific antibody activates CB1954 selectively at the tumour site. Given its high specific activity for CB1954 the *E. coli* nitroreductase is an attractive enzyme for ADEPT.

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