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THE DIFFERENCES IN KINETICS OF RAT AND HUMAN DT DIAPHORASE RESULT IN A DIFFERENTIAL SENSITIVITY OF DERIVED CELL LINES TO CB 1954 (5-(AZIRIDIN-1-YL)-2,4-DINITROBENZAMIDE)

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Abstract—DT diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2) isolated from Walker 256 rat carcinoma cells can convert CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) to a cytotoxic DNA interstrand cross-linking agent. This is achieved by reduction of the 4-nitro group of CB 1954 to produce the hydroxylamino species, a bioactivation which accounts for the much greater sensitivity of Walker cells to CB 1954 when compared with other cells which are unable to carry out this reduction (Knox *et al.*, *Biochem Pharmacol* **37**: 4661–4669 and 4671–4677, 1988). As predicted from their measured DT diaphorase activities a number of rat hepatoma and hepatocyte cell lines were also shown to be sensitive to CB 1954. However, no CB 1954-sensitive cell lines of human origin were found, although levels of DT diaphorase similar to those in the sensitive rat cells were present in these cells. The human cells were as sensitive as rat cells to the active form of CB 1954 (5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide). DT diaphorase, purified to homogeneity from human Hep G2 cells, did metabolize CB 1954 to this 4-hydroxylamino product, but the rate of CB 1954 reduction and thus production of the cytotoxic product, was much lower than that of purified Walker enzyme (ratio of K_{cat} =6.4). In addition, CB 1954 could be considered an inhibitor of, rather than a substrate for, the human form of DT diaphorase. The purified rat and human DT diaphorases possessed otherwise similar biochemical and molecular properties. These findings explain the decreased sensitivity towards CB 1954 of human cell lines when compared to rat cell lines.

The selective cytotoxicity of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) towards Walker 256 rat carcinoma cells has been accounted for by its ability to act as a difunctional agent by forming DNA interstrand crosslinks in Walker but not in non-toxically affected cells [1]. Chemically, CB 1954 can only react as a monofunctional alkylating agent (by virtue of its single aziridine group) and the observed cross-link formation is a result of the bioreduction of the drug to a difunctionally-reacting species. This has been identified as the 4-hydroxylamino derivative, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide [2]. The enzyme responsible for the aerobic reduction of CB 1954 in Walker cells has been identified as a form of NAD(P)H dehydrogenase (quinone) (DT diaphorase, EC 1.6.99.2) [2, 3]. This enzyme catalyses the reduction of the 4-nitro group to the hydroxylamine product, which is toxic to cells resistant to CB 1954 and produces cross-links in their DNA. The enzyme, commonly called DT diaphorase, has been isolated, purified and characterized by comparison of partial protein sequences, coenzymes, substrate and inhibitor kinetics and spectroscopic data. DT diaphorase is a flavoprotein which normally promotes the two electron reduction of quinones to hydroquinones, protecting cells against the damaging effects of oxygen species generated during the cycling of quinone radicals by one electron reduction reactions (for a review see Ref. 4). We have previously shown that inhibitors of this enzyme, while potentiating the cytotoxicity of menadione, protect against the cytotoxicity and DNA cross-linking ability of CB 1954 in Walker cells by inhibiting its bioactivation [5].

The dramatic inhibitory effect on the growth of the Walker tumour *in vivo* [6] raised the possibility that similarly responsive human tumour types could be found. Thus, CB 1954 has been described as "a drug in search of a human tumour to treat" [7]. The identification and characterization of the enzyme which reduces CB 1954 to the toxic cross-linking derivative (5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide), renewed the possibility of identifying human tumour types with similar sensitivity to the Walker carcinoma. This was because this enzyme, DT diaphorase, is known to be widely distributed in mammalian tissues (see Ref. 4) and its activity is considerably elevated in chemically-induced preneoplastic nodules in rat liver when compared with normal tissue [8]. The enzyme is also selectively induced in the livers of tumour-bearing animals [9, 10]. In addition, DT diaphorase activity is present in a variety of tissues and cell lines of human origin. High activity has been detected in stomach and abdominal adipose tissue [11, 12] and elevated activity has been reported in human tumour cell lines of breast, brain [13] and liver origin [14]. There is a marked increase in the activity of DT diaphorase in human colonic carcinomas when compared with the enzymatic activity of the surrounding normal colonic mucosa [15].

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No significant differences between the Walker, rat liver and human forms of this enzyme have been reported. Both the rat and human forms of DT diaphorase have been cloned and sequenced [16, 17]. Human DT diaphorase cDNA and protein are 83% and 85% homologous with the rat liver cytosolic cDNA and protein, respectively [17]. Both are inducible cytosolic flavoproteins encoded by a single gene. The human protein is biochemically very similar to the rat protein, only small differences between K_m values for the substrates menadione and NADH having been found [12]. Thus, one might therefore predict that human or rat DT diaphorase would metabolize CB 1954 in a manner similar to the protein from Walker cells. Thus, the cytotoxicity resulting from the bioactivation of CB 1954 might be observed in human tumours expressing significant levels of this enzyme.

To investigate this possibility, we have determined the cytotoxicity of CB 1954 in both rat and human cell lines with comparable DT diaphorase activities to the Walker cell line. We have also purified the human enzyme to homogeneity and compared the kinetics and metabolism of CB 1954 by this form of DT diaphorase with those of the Walker form of the enzyme.

MATERIALS AND METHODS

Materials. Tissue culture media and foetal calf serum were supplied by Gibco Ltd (Paisley, U.K.). All other chemicals and reagents were supplied by the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. Cyanogen bromide activated Sepharose 4B was obtained from Pharmacia LKB (Milton Keynes, U.K.). Hybond-C Extra nitrocellulose and protein molecular weight markers were obtained from Amersham (Aylesbury, U.K.). JB1 and BL8L cell lines were kindly supplied by Dr T. A. Connors (MRC Toxicology Unit, Carshalton, U.K.). The cell line, HTC, was a gift from Ms A. Redmond (Dublin City University, Eire). CB 1954 and its derivatives were synthesized or supplied by Dr M. Jarman (ICR).

Determination of the effects of compounds on cell survival. For cell lines growing in monolayer, 25-cm^2 flasks were seeded in duplicate for each drug concentration with 5×10^4 cells per mL in 5 mL of their appropriate growth media and incubated (48–72 hr) at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Cells were then treated for 2 hr with the drug in fresh media under the same culture conditions. The treated cells were then washed with fresh media, trypsinized and plated out in triplicate at various concentrations. After 15 to 21 days incubation under standard conditions, colonies were fixed and stained. Where required for clonal growth, lethally irradiated 3T3 mouse fibroblast feeder cells were plated out at a concentration of 1×10^3 cells/mL in 5 mL of media 24 hr before treated cells were plated out.

Enzyme activity and kinetic studies. DT diaphorase activity was assayed as previously described [3] employing menadione as substrate and cytochrome *c* as terminal electron acceptor. Normally, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0, was employed as a buffer. Activity was defined as the cytochrome *c* reduction inhibited by 1 μM dicoumarol and expressed as nmoles cytochrome *c* reduced per minute per mg of protein. K_m values were determined from Eadie–Hoftsee plots and inhibition constants (K_i and K_i' values) and the types of inhibition were determined from Dixon plots, also as described previously [5].

Preparation of cell extracts for enzyme assays. Cells were grown to sub-confluence in 175 cm^2 tissue culture flasks, trypsinized and the pellet obtained washed several times in ice-cold PBS. The cells were resuspended in PBS at a concentration of 5×10^6 cells per mL and aprotonin added to a final volume of 1%. The suspension was sonicated and centrifuged at 4° to pellet the cell debris. The supernatant obtained was filtered through 0.2 μm filters. Protein concentration was determined by a conventional assay (BioRad) and calibrated against a range of bovine serum albumin standards.

Purification of DT diaphorase from Walker tumour cells. The purification of this enzyme has been previously described [2].

Preparation of anti-DT diaphorase polyclonal antibody. Rabbit polyclonal antisera was raised against Walker cell DT diaphorase by Dr C. Dean (ICR). The antisera was purified on a cyanogen bromide-activated Sepharose 4B column which had been chemically linked with purified Walker DT diaphorase. Purified antibody was eluted with 3 M potassium thiocyanate and subsequently dialysed against an appropriate buffer.

Purification of DT diaphorase from Hep G2 cells. Human hepatoblastoma Hep G2 cells were grown to confluence on tissue culture plates (245 mm × 245 mm) at 37° in Dulbecco's modified Eagle's medium plus 10% foetal calf serum and 10 μg/mL insulin. Cells were harvested by trypsinization and the pellet obtained washed three times in ice-cold sterile PBS. The cell pellet was then resuspended in PBS and aprotinin was added to a final concentration of 1.0%. The cells were disrupted by sonication, each 20 sec pulse being intervened by a rest period on ice. After centrifugation at 150,000 *g* (30 min, 4°), the supernatant was filtered successively through 0.45 and 0.2 μm sterile filters. The filtered supernatant was injected onto a TSK G3000 SWG (21.5 × 600 mm) gel filtration column with a 2 mL loop, and eluted at 0.5 mL/min with 0.01 M sodium phosphate buffer (pH 7). Fractions (0.5 mL) were collected and placed on ice. All buffer reservoirs were kept chilled on ice throughout the purification. Each fraction was assayed for DT diaphorase activity by the standard assay described above and CB 1954 metabolizing activity was determined by HPLC. Fractions containing the appropriate activity were pooled and 1-mL aliquots loaded onto a cyanogen bromide activated Sepharose 4B column at 4° which had been chemically-linked with purified anti-Walker cell DT diaphorase polyclonal antibody. The column was washed with 10 mM sodium phosphate buffer (pH 7) containing 0.02% sodium azide until no protein was detected in the effluent. The enzyme was then eluted at 0.333 mL/min in 10 mM sodium phosphate buffer

(pH 7) containing 1.5 M NaCl, 50 μM FAD and 1 mM NADH. Bovine serum albumin was present at a final concentration of 0.01%. Fractions were assayed for enzyme activity as above. Active fractions were desalted (on a Pharmacia PD10 column) pooled and concentrated by centrifugation (Amicon, centricon 10), desalting further with elution buffer containing no NaCl or NADH. Each stage of the purification was monitored by electrophoresis of the resulting protein(s) through duplicate SDS–polyacrylamide gels for subsequent location by either staining with Coomassie blue or by Western blotting.

Electrophoresis and Western blotting. SDS–PAGE was carried out on either 12% or 15% gels as described by Laemmli [18]. Duplicate gels were blotted onto Hybond-C Extra membranes as described by Towbin *et al.* [19]. Membranes were blocked in a solution containing 50 mM Tris, 80 mM NaCl, 2% BSA and 5% dry milk (Marvel) pH 8.0 at 37° and then washed three times after each incubation with PBS containing 0.1% Tween 20. All incubations were at 37° for 2 hr. Purified primary antibody was routinely used at a concentration of 2–4 μg/mL in PBS containing 5% BSA. ^{125}I-Labelled sheep anti-rabbit F(ab)$_2$ was employed as second antibody at 10^5 cpm/mL in PBS containing 0.5% BSA. Autoradiograms were exposed at −70° for 1–7 days.

CB 1954 metabolism studies. The enzymatic reduction of CB 1954 by purified Walker and Hep G2 DT diaphorase was followed by HPLC. CB 1954 (100 μM and also containing [U-^{3}H]CB 1954 at 1.6×10^5 cpm per nmole), NADH (500 μM) were incubated with enzyme in 10 mM sodium phosphate buffer (pH 7) in air at 37°. At various times aliquots (10 μL) were injected onto a Partisphere SCX (110 × 4.7 mm) HPLC column and eluted isocratically (1 mL/min) with 100 mM NaH_2PO_4. The elutate was continuously monitored for absorption at 310, 260 and 360 nm and the spectra of eluting components recorded using a diode-array detector. Samples (0.25 mL) were collected and the tritium activity of each determined by liquid scintillation counting. This separation system could resolve all the expected reduction products as indicated in Fig. 4.

To further confirm the identity of any reduction products, the above reaction mixture was also injected onto an ODS-5 reverse phase HPLC column and eluted (1 mL/min) with a methanol gradient (0–40% linear over 30 min, 40–100% linear over 10 min).

RESULTS

Cell survival data and NAD(P)H dehydrogenase activities

The following cell lines were employed: of rat origin, BL8L, an established but non-malignant cell line derived from hepatocytes [20]; JB1, a malignant (chemically-induced) cell line arising from hepatocyte cultures [21] and HTC, an 'epithelioid-like' cell line derived from primary culture of the ascites form of an experimental rat hepatoma [22]. Of human origin, Hep G2, a hepatoblastoma cell line [23], MCF-7 and SW 837, tumour lines of breast [24] and colon [25]

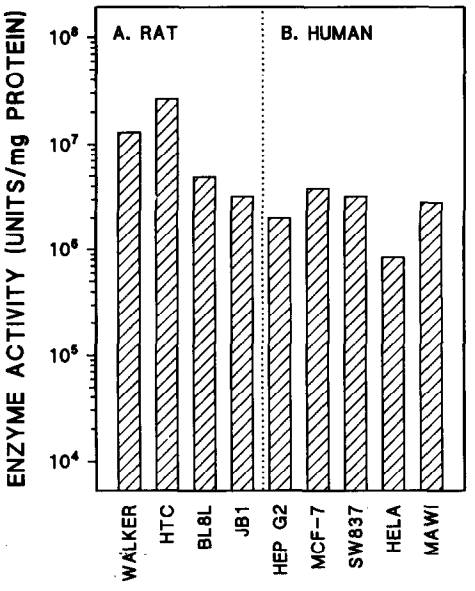

Fig. 1. The activity of the enzyme DT diaphorase in cell lines of either (A) rat or (B) human origin. DT diaphorase activity was assayed employing menadione (10 μM) as substrate and cytochrome *c* (70 μM) as a terminal electron acceptor. Activity was defined as the cytochrome *c* reduction inhibited by 1 μM dicoumarol and expressed as nmoles cytochrome *c* reduced per minute (units) per mg of total protein at 37°.

origin, respectively, and a HeLa cell subline. The DT diaphorase activities present in all these cell lines are shown in Fig. 1. DT diaphorase activity was defined as the menadione (cytochrome *c*) reduction inhibited by 1 μM dicoumarol and expressed as nmoles cytochrome *c* reduced per minute (units) per mg of total protein at 37°. In general, the human cell lines possessed similar DT diaphorase activity to the rat (and Walker) cell lines. The cytotoxic effects of these cell lines following treatment with CB 1954 are shown in Fig. 2A. The rat cell lines were all sensitive to CB 1954 and the resulting cell kill after a 2 hr exposure to the drug approached that obtained in Walker cells. Human cell lines were, on the other hand, all dramatically less toxically effected by CB 1954 with over a 100-fold higher dose of the agent being required to produce a comparable cytotoxic response to that obtained in cells of rat origin. However, the other human cell lines were more sensitive to CB 1954 than the HeLa subline (which expressed the lowest amounts of DT diaphorase activity). The response of both the rat and human cell lines to the active form of CB 1954, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is shown in Fig. 2B. In contrast to the large difference in their cytotoxic response towards CB 1954, both the rat and the human cell lines were similarly affected by the 4-hydroxylamino derivative of CB 1954.

Purification of DT diaphorase from human Hep G2 cells

Gel filtration chromatography on a TSK G3000

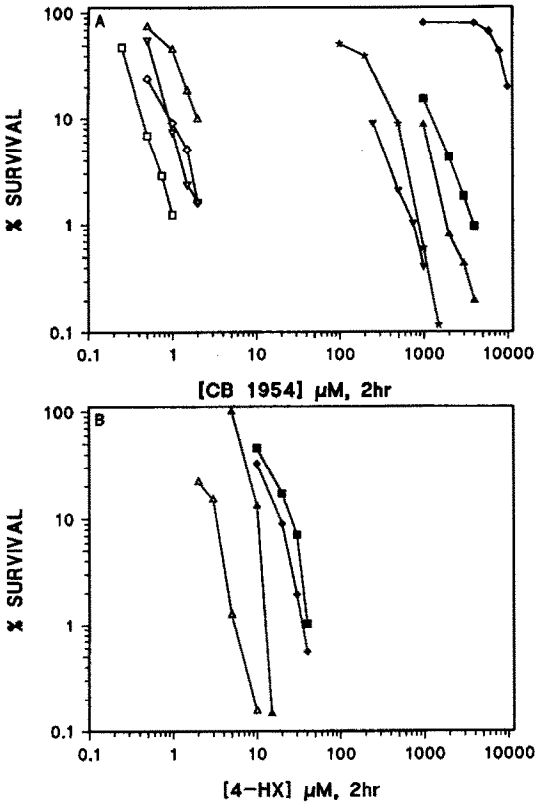

Fig. 2. The effect of (A) CB 1954 or (B) 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide on the survival of cells of either rat or human origin. The rat cell lines used were: BL8L (▽); JB1 (◇); HTC (△) and Walker (□). The human cell lines were: Hep G2 (■); MCF-7 (▲); SW 837 (▼); Mawi (★) and HeLa (◆). Cells were exposed to the agent for 2 hr at 37° and then assayed for colony forming ability. Drug concentrations are plotted on a logarithmic scale.

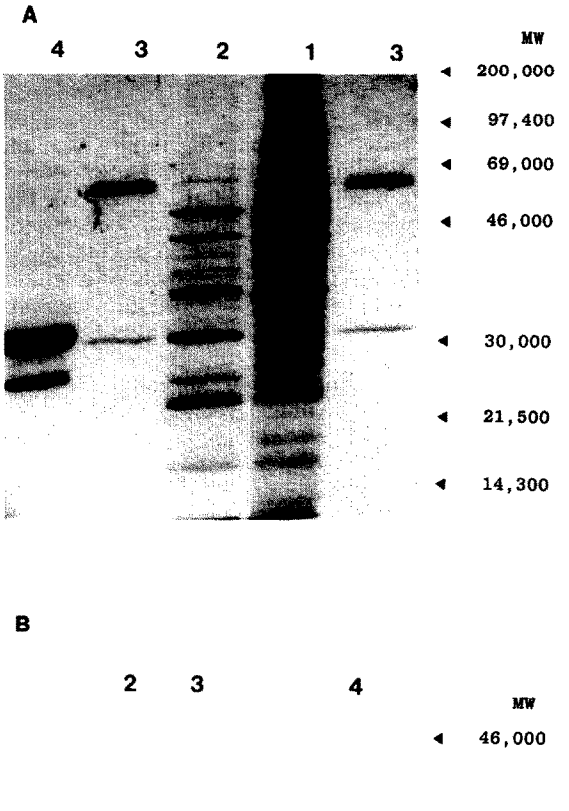

Fig. 3. The purification of DT diaphorase from human, Hep G2, cells. The steps involved in the purification are illustrated by electrophoresis of the resulting protein(s) through SDS–polyacrylamide gels and their subsequent location by (A) Coomassie blue staining (12% gel) or (B) Western blotting (15% gel) with an antibody raised against the Walker form of DT diaphorase. (1) Crude extract, (2) after the gel filtration step, (3) after affinity purification and (4) the Walker form of DT diaphorase. The upper band seen in the Coomassie blue staining of the human enzyme is BSA added to stabilize the human form of DT diaphorase.

column resolved DT diaphorase from Hep G2 cells as a single peak without loss of activity. The apparent molecular weight of the protein estimated by this gel filtration chromatography step, was only 4.0 kDa, suggesting some electrostatic interactions between the column material resulting in an ambiguously high retention time but a satisfactory separation (Fig. 3). The protein was found to cross-react with polyclonal antibodies raised against the purified Walker enzyme (Fig. 3B) and was therefore preferentially retained on an antibody affinity column. SDS–PAGE (Fig. 3A) and subsequent Western blotting (Fig. 3B) of the eluted fractions revealed a single protein band with an estimated molecular weight of 31 kDa. The presence of FAD and BSA were found to enhance stability and therefore recovery of the protein under the salt elution conditions. The above procedure provided a 227-fold purification of the enzyme to homogeneity between the first and final purification steps.

The enzymatic reduction of CB 1954 by DT diaphorase from Hep G2 cells

Under the same reaction conditions both the Walker and Hep G2 forms of DT diaphorase reduced CB 1954 to a single product (Fig. 4) and this was shown to be 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide by comparison of retention times and spectral characteristics. Its identity was further confirmed by chromatography using an alternative separation system (data not shown). However, as indicated in Fig. 4, the human form of DT diaphorase reduced less CB 1954 and produced less 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide than the

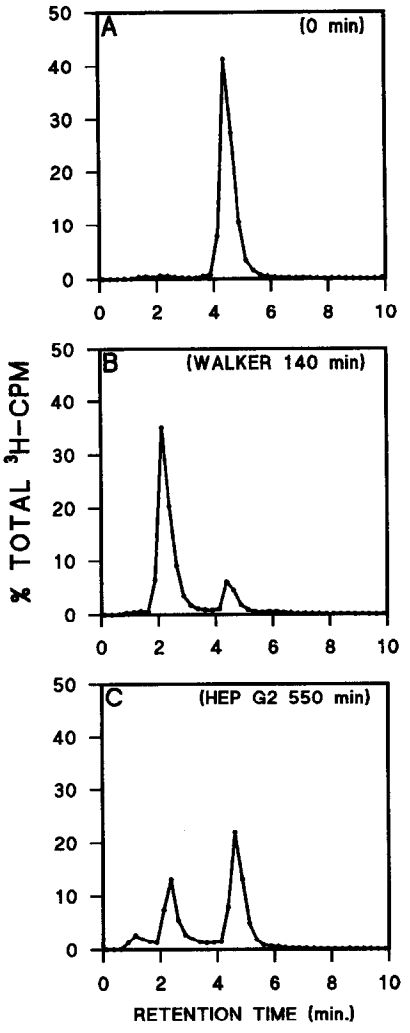

Fig. 4. The products generated by the aerobic reduction of CB 1954 by 2×10^8 units/mL of DT diaphorase for (A) 0 min; (B) 140 min with the Walker (rat) form of the enzyme; or (C) 550 min with the Hep G2 (human) form of DT diaphorase. Reduction conditions are as described in the text, initial concentrations of CB 1954 and NADH were 100 and 500 μM, respectively. Elution times of the standards were: CB 1954, 4.2 min; 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide, 3.2 min; 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, 2.1 min; 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide, 6.1 min and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide, 3.7 min.

same amount of the Walker form of the enzyme. As shown in Fig. 5, under the standard conditions used, the rate of reduction of CB 1954 by the Hep G2 (human) enzyme was approximately 10-fold lower when compared with the Walker (rat) enzyme. Thus, CB 1954 is not readily reduced by the human form of DT diaphorase. This difference was not due to lack of stability of the human enzyme, as the ability of the reaction mixture to reduce menadione was retained (data not shown).

Kinetic data for DT diaphorase from Hep G2 or Walker cells

Kinetic parameters for DT diaphorase purified

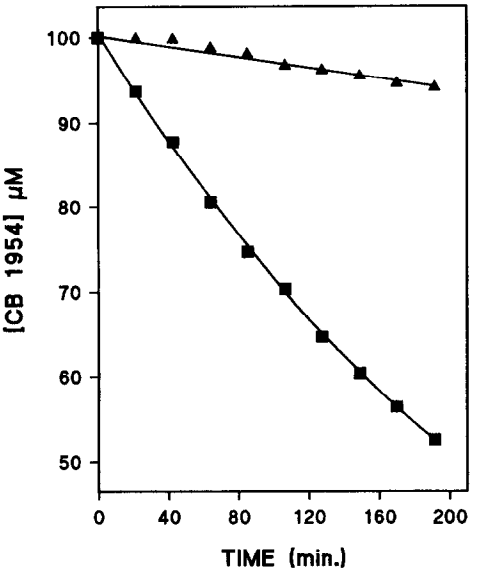

Fig. 5. The rate of reduction of CB 1954 by 6×10^7 units/mL of either the Walker (rat) (■) or Hep G2 (human) (▲) forms of DT diaphorase. Reduction conditions are as described in the text, initial concentrations of CB 1954 and NADH were 100 and 500 μM, respectively.

from either human Hep G2 cells or Walker rat cells are given in Table 1. In general, with the exception of those parameters referring to CB 1954, the kinetic parameters of the Hep G2 and Walker forms of DT diaphorase were similar. Thus, K_m values with respect to menadione, NADPH and NADH plots were found to be 3.1, 85 and 222 μM, respectively, for the Hep G2 enzyme and 1.3, 71 and 78 μM for the Walker form of DT diaphorase. The limiting velocities at unit enzyme concentration (K_{cat}) were also similar (Hep G2 6.1×10^7 min^{-1}, Walker 6.5×10^7 min^{-1}).

The kinetic constants and mechanism of inhibition by dicoumarol and caffeine were also determined (Table 2). Inhibition by dicoumarol was found to be mixed with respect to NADH for both forms of the enzyme, with values for K_i and K_i', being 1.4 and 4.0 nM, respectively, for the Hep G2 form and 10 and 20 nM for the Walker form of DT diaphorase. Caffeine also inhibited both forms of the enzyme although the type of inhibition was different between the two DT diaphorase forms.

In contrast to the similar kinetic parameters with respect to NAD(P)H and menadione, a marked difference was observed between the kinetic parameters of the Hep G2 and Walker forms of DT diaphorase with respect to CB 1954. Although the K_m values towards CB 1954 were similar for the human and Walker forms of DT diaphorase (1403 and 826 μM, respectively), there was a notable difference in the respective K_{cat} values (0.64 and 4.1 min^{-1}). Thus, the Hep G2 form of DT diaphorase intrinsically reduces CB 1954 at less than a sixth of the rate of the Walker enzyme. Menadione reduction by both forms of DT diaphorase was inhibited by CB 1954. With respect to the Walker form of the

Table 1. Kinetic parameters for the Hep G2 (human) and Walker (rat) forms of DT diaphorase

Compound		DT diaphorase purified from Hep G2	Walker
NADH	K_m	222 μM	78 μM
NADPH	K_m	85 μM	71 μM
Menadione	K_m	3.1 μM	1.3 μM*
	K_{cat}	6.1×10^7 min^{-1}	6.5×10^7 min^{-1}
CB 1954	K_m	1403 μM	826 μM
	K_{cat}	0.64 min^{-1}	4.1 min^{-1}

* Data from Ref. 2.

Table 2. The ability of various compounds to inhibit the reduction of menadione by either Hep G2 (human) or Walker (rat) DT diaphorase

Inhibitor	Varying	Hep G2 K_i	Hep G2 K_i'	Hep G2 Type	Walker* K_i	Walker* K_i'	Walker* Type
Dicoumarol	NADH	1.4 nM	4.0 nM	Mixed	10 nM	20 nM	Mixed
	Menadione	1.9 nM†	4.4 nM	Mixed	10 nM		Noncompetitive
Caffeine	NADH	—	1.25 mM†	Uncompetitive	1.08 mM	—	Competitive
	Menadione	233 μM	—	Competitive	0.66 mM	3.15 mM	Mixed
CB 1954	NADH	130 μM	—	Competitive	—		Complex
	Menadione	606 μM	714 μM	Mixed	—		

All measurements were carried out at 37° and the standard concentrations of menadione and NADH were 10 and 500 μM, respectively.

* Data from Ref. 5.

† Standard deviations > ±5%.

enzyme the type of inhibition was complex as the predicted linear result was not obtained when the reciprocal of the rate of reduction was plotted against the concentration of CB 1954 (data not shown). CB 1954 was a competitive inhibitor (with respect to NADH) of menadione reduction by the Hep G2 form of DT diaphorase with a K_i value determined to be 130 μM.

Increasing concentrations of NaCl were found to inhibit enzyme activity which could be recovered in the presence of FAD (data not shown). This suggested that the flavin group may be more weakly associated with the apoprotein than in the Walker cell enzyme. Low concentrations of FAD (1 μM) were also found to enhance the dicoumarol-inhibitable menadione-reducing activity from crude cell extracts of Hep G2 cells.

DISCUSSION

A dramatic sensitivity towards the monofunctional alkylating agent CB 1954 is a consequence of its reduction to a difunctional compound that can induce DNA cross-links. The active form of CB 1954 has been identified as 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide [2]. This compound can produce DNA cross-links in cells insensitive to CB 1954 but not in naked DNA [2] and a further activation step is involved that further converts 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to the proximal DNA cross-linking species. A second activation step such as this would be analogous to that found for the activation of the hydroxylamines formed by the metabolism of 4-nitroquinoline - *N* - oxide and *N*-acetylaminofluorene [26, 27]. Formation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is a result of the reduction of CB 1954. In Walker cells this reduction is performed in air by the enzyme DT diaphorase [2, 3] and expression of this enzyme fully accounts for the selective toxicity of CB 1954 in cells of the Walker tumour.

As we have discussed in the introduction, identification of this enzyme, which is known to be raised in many tumours, suggested that other tumours apart from the Walker tumour could be sensitive to CB 1954. A number of rat cell lines are sensitive to CB 1954 as one might predict from their levels of the activating enzyme, DT diaphorase. Thus, the Walker tumour is not uniquely sensitive towards CB 1954. However, the response of a range of human cell lines to CB 1954 with equivalent DT diaphorase activity to these sensitive rat cell lines was also examined but all were found to be much less sensitive to the agent. Thus, human tumour cell lines possess significant levels of DT diaphorase, but lack the sensitivity towards CB 1954 that the equivalent level of enzyme in a rat cell line would confer.

This decreased sensitivity towards CB 1954 of

human cells could be accounted for by the following reasons:

(a) Human cells could be resistant towards or rapidly repair the DNA adducts formed from the bioactivation of CB 1954.

(b) There could be a failure to further activate the 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to the proximal DNA cross-linking species.

(c) Other enzymes may be present in human cells capable of detoxifying CB 1954 by alternative metabolic pathways.

(d) The human form of DT diaphorase may not reduce CB 1954, or reduce it to a less cytotoxic product than the 4-hydroxylamino derivative.

The human cells were found to be of similar sensitivity towards the activated form of CB 1954, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, as the rat cell lines. Thus, failure to further activate the 4-hydroxylamine product or resistance to the DNA adducts formed in human cell lines could not be the mechanism by which these human cell lines lack the expected sensitivity to CB 1954.

In order to investigate the other proposed mechanisms outlined above, it was necessary to purify the human form of the enzyme. DT diaphorase was purified to homogeneity from Hep G2 cells in a two-step purification, an initial gel filtration step followed by antibody-affinity chromatography, the antibody being raised against the Walker form of DT diaphorase. Partial purification of the human enzyme from sources such as kidney, brain and testis has been reported [28, 29]. The presence of FAD and NADH have been utilized to stabilize the protein owing to poor yields and limited knowledge about the nature of the human enzyme [28] and BSA has also been reported to stabilize DT diaphorase [30]. The presence of NADH could improve its thermostability [28], whereas the flavin group, which is released at high ionic strength, may be retained in the presence of excess FAD. Thus, FAD, BSA and NADH were included in the purification scheme of the enzyme from Hep G2 cells as the stability of the protein in its pure form had been questioned, although a successful purification scheme for the enzyme from abdominal adipose tissue without the above additions has been described [12]. This purification system employed azodicoumarol affinity and anion exchange chromatography. Using our purification system DT diaphorase was purified to homogeneity from Hep G2 cells. However, concentration of the human enzyme resulted in some loss of menadione-reducing activity but this could be partially recovered by addition of FAD. Therefore the pure protein was stabilized by addition of the above factors. The molecular weight of the human protein has been reported to be 32 kDa [12] (30,880 daltons from the cDNA sequence [17]), which is smaller than the values reported for the rat (32–55 kDa [4] and the Walker enzyme (33.5 kDa) [2], although both human and rat genes code for a protein of 274 residues [16, 17]. In close agreement with this, the molecular weight obtained for Hep G2 DT diaphorase was 31 kDa. That there is a difference in apparent molecular weight between the Walker and human forms of the enzyme is illustrated in Fig. 3B where both forms of the enzyme have been blotted from the same gel. This figure also demonstrates that a polyclonal antibody raised against the purified Walker enzyme can cross-react with Hep G2 DT diaphorase. Previous reports have also demonstrated the cross-reactivity of antibodies raised against rat DT diaphorase with the human enzyme [14].

The enzyme, DT diaphorase, has generally been regarded as a detoxifying enzyme, catalysing the two electron reduction of quinones and quinoid compounds including vitamin K, to hydroquinones [4]. Although DT diaphorase has been extensively studied, the most common source of the enzyme is rat liver and information regarding the human protein is limited. As might be predicted from the large degree of homology between the rat and human forms of DT diaphorase the biochemical properties of the Hep G2 and Walker forms of the enzyme with respect to the reduction of menadione are very similar. Our values for the K_m of the human, Hep G2 form of the enzyme, with respect to menadione and NADH are in close agreement with values obtained with the human enzyme, as isolated from abdominal adipose tissue [12].

However, in contrast to these similar biochemical properties with respect to NAD(P)H and menadione, significant differences were observed in the ability of DT diaphorase isolated from either Hep G2 or Walker cells in their ability to reduce CB 1954 to the active 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide derivative. Although, under the reaction conditions used, both forms of the enzyme produced 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. The absolute rate of reduction of CB 1954 (i.e. under saturating conditions of both NADH and CB 1954) is the K_{cat} value. This is over six-fold higher for the Walker cell form of the enzyme ($4.1\ min^{-1}$) than for the human DT diaphorase ($0.64\ min^{-1}$). As stated these values refer to saturating conditions of both CB 1954 and NADH and it should be noted that these saturating concentrations will probably not be achieved intracellularly. As the K_m values of the Hep G2 DT diaphorase for both CB 1954 and NADH are higher than those for the Walker form of the enzyme, the further the actual concentrations of CB 1954 and NADH are away from those required to saturate the enzyme, the greater the difference between the reduction rates of CB 1954 by the Walker and Hep G2 forms will become. Thus, it can be estimated that as the substrate concentrations decrease below 100 μM, there will be a 12–15-fold difference in the relative rates of CB 1954 reduction and consequently 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide production. This intrinsic difference in the ability of the human Hep G2 DT diaphorase to produce the required cytotoxic species from CB 1954 would account for the lack of sensitivity of human cells towards this agent.

The reason for the observed differences between the human and Walker forms of DT diaphorase, with respect to their reduction of CB 1954 but not

other substrates, is not obvious. The proposed pyrophosphate binding region for NAD(P)H in the rat enzyme [31] is fully conserved with the human form DT diaphorase (although the K_m values for NADH differ significantly). However, we have observed that there is a change of charge (amino acids 124) within the proposed substrate binding region (amino acids 96–132) [32]. This may be of significance regarding the difference in rates of reduction of CB 1954 between the human and the rat enzymes. It is also of interest to note that CB 1954 inhibits the reduction of menadione by DT diaphorase. Although parameters could not be obtained for the Walker enzyme, CB 1954 is a competitive inhibitor (with respect to NADH) of the Hep G2 enzyme. The K_i value (130 μM) indicates that CB 1954 can bind reasonably well to the enzyme (much better than might be inferred from the K_m of the enzyme for CB 1954) and CB 1954 could be considered an inhibitor of, rather than a substrate for, human DT diaphorase. This observation parallels the recent finding that mitomycin C is not metabolized but is an inhibitor of semipurified human kidney DT diaphorase [29].

In summary, our initial observations that the sensitivity of Walker cells towards CB 1954 is due to the activation of this compound to a difunctional form by the enzyme DT diaphorase, raised the possibility that other cell types, expressing this enzyme, could also be sensitive to this compound. Similar sensitivity has now been demonstrated in a number of rat cell lines with significant DT diaphorase activity. However, human cell lines with similar DT diaphorase activities were not sensitive to CB 1954. The cell lines employed were sensitive to the active form, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. The reason that human cells lack the sensitivity towards CB 1954 inferred by their DT diaphorase activity is because the human form of DT diaphorase is intrinsically less able to reduce this compound. Thus, DT diaphorase, an important cytosolic detoxifying enzyme which is responsible for the cytotoxic activation of a drug in rat cells, does not readily activate it in human cells. The observation emphasizes that rodent systems may not always be suitable models for the development of drugs for human therapy.

Although CB 1954 may not have a place in the chemotherapy of human tumours it is conceivable that a derivative of this compound could be found that would be more readily activated by the human form of DT diaphorase. It is possible that such a bioactivatable compound could regenerate in specific human tumours the exquisite sensitivity first observed with CB 1954 in the Walker tumour over 20 years ago.

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REFERENCES

1. Roberts JJ, Friedlos F and Knox RJ, CB 1954 (2,4-dinitro-5-aziridinyl benzamide) becomes a DNA interstrand crosslinking agent in Walker tumour cells. *Biochem Biophys Res Commun* **140**: 1073–1078, 1986.
2. Knox RJ, Friedlos F, Jarman M and Roberts JJ, A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. *Biochem Pharmacol* **37**: 4661–4669, 1988.
3. Knox RJ, Boland MP, Friedlos F, Coles B, Southan C and Roberts JJ, 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). *Biochem Pharmacol* **37**: 4671–4677, 1988.
4. Ernster L, DT diaphorase: a historical review. *Chemica Scripta* **27A**, 1–13, 1987.
5. Roberts JJ, Marchbank T, Kotsaki-Kovatsi VP, Boland MP, Friedlos F and Knox RJ, Caffeine, aminoimidazolecarboxamide and dicoumarol, inhibitors of NAD(P)H dehydrogenase (quinone) (DT diaphorase), prevent both the cytotoxicity and DNA interstrand crosslinking produced by 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) in Walker cells. *Biochem Pharmacol* **38**: 4137–4143, 1989.
6. Cobb LM, Connors TA, Elson LA, Khan AH, Mitchley BCV, Ross WCJ and Whisson ME, 2,4-Dinitro-5-ethyleneiminobenzamide (CB 1954): a potent and selective inhibitor of the growth of the Walker carcinoma 256. *Biochem Pharmacol* **18**: 1519–1527, 1969.
7. Workman P, Morgan JE, Talbot K, Wright KA, Donaldson J and Twentyman PR, CB 1954 revisited II. Toxicity and antitumour activity. *Cancer Chemother Pharmacol* **16**: 9–14, 1986.
8. Schor NA, Ogawa K, Lee G and Farber E, The use of the D-T diaphorase for the detection of foci of early neoplastic transformation in rat liver. *Cancer Lett* **5**: 167–171, 1978.
9. Pickett CB, Williams JB, Lu AYH and Cameron RG, Regulation of glutathione transferase and DT-diaphorase mRNAs in persistent hepatocyte nodules during chemical hepatocarcinogenesis. *Proc Natl Acad Sci USA* **81**: 5091–5095, 1984.
10. Beyer RE, Segura-Aguilar JE and Ernster L, The anticancer enzyme DT diaphorase is induced selectively in liver during ascites hepatoma growth. *Anticancer Res* **8**: 233–238, 1988.
11. Martin LF, Patrick SD and Wallin R, DT-diaphorase in morbidly obese patients. *Cancer Lett* **36**: 341–347, 1987.
12. Smith D, Martin LF and Wallin R, Human DT-diaphorase, a potential cancer protecting enzyme. Its purification from abdominal adipose tissue. *Cancer Lett* **42**: 103–112, 1988.
13. Berger MS, Talcott RE, Rosenblum ML, Silva M, Ali-Osman F and Smith MT, Use of quinones in brain-tumor therapy: preliminary results of preclinical laboratory investigations. *J Toxicol Environ Health* **16**: 713–719, 1985.
14. Beyer RE, Segura-Aguilar J, Lind C and Castro VM, DT diaphorase activity in various cells in culture with emphasis on induction in ascites hepatoma cells. *Chemica Scripta* **27A**: 145–150, 1987.
15. Schor NA and Cornelisse CJ, Biochemical and quantitative histochemical study of reduced pyridine nucleotide dehydrogenation by human colonic carcinomas. *Cancer Res* **43**: 4850–4855, 1983.

16. Robertson JA, Chen H and Nebert DW, NAD(P)H: menadione oxidoreductase. Novel purification of enzyme, cDNA and complete amino acid sequence, and gene regulation. *J Biol Chem* **261**: 15794–15799, 1986.
17. Jaiswal AK, McBride OW, Adesnik M and Nebert DW, Human dioxin-inducible cytosolic NAD(P)H: Menadione oxidoreductase: cDNA sequence and localization of gene to chromosome 16. *J Biol Chem* **263**: 13572–13578, 1988.
18. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
19. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
20. Judah DJ, Legg RF and Neal GE, Development of resistance to cytotoxicity during aflatoxin carcinogenesis. *Nature* **265**: 343–345, 1977.
21. Manson MM, Legg RF, Watson JV, Green JA and Neal GE, An examination of the relative resistances to aflatoxin B1 and susceptibilities to gamma-glutamyl p-phenylene diamine mustard of gamma-glutamyl transferase negative and positive cell lines. *Carcinogenesis* **2**: 661–670, 1981.
22. Thompson EB, Tomkins GM and Curran JE, Induction of tyrosine a-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. *Proc Natl Acad Sci USA* **56**: 296–303, 1966.
23. Aden DP, Fogel A, Plotkin S, Damjanov I and Knowles BB, Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* **282**: 615–616, 1979.
24. Soule HD, Vazquez J, Long A, Albert S and Brennan M, A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* **51**: 1409–1416, 1973.
25. Leibovitz A, Stinson JC, McCombs III WB, McCoy CE, Mazur KC and Mabry ND, Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* **36**: 4562–4569, 1976.
26. Tada M and Tada M, Seryl-*t*-RNA synthetase and activation of the carcinogen 4-nitroquinoline 1-oxide. *Nature* **255**: 510–512, 1975.
27. DeBaum JR, Miller EC and Miller JA, *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein-(methion-*S*-yl) binding in rat liver. *Cancer Res* **30**: 577–595, 1970.
28. Edwards YH, Potter J and Hopkinson DA, Human FAD-Dependent NAD(P)H Diaphorase. *Biochem J* **187**: 429–436, 1980.
29. Schlager JJ and Powis G, Mitomycin C is not metabolized by but is an inhibitor of human kidney NAD(P)H: (quinone-acceptor) oxidoreductase. *Cancer Chemother Pharmacol* **22**: 126–130, 1988.
30. Prochaska HJ and Talalay P, Purification and characterisation of two isofunctional forms of NAD(P)H: quinone reductase from mouse liver. *J Biol Chem* **261**: 1372–1378, 1986.
31. Liu X-F, Yuan H, Haniu M, Iyanagi T, Shively JE and Chen S, Reaction of rat liver DT diaphorase (NAD(P)H: quinone acceptor reductase) with 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine. *Mol Pharmacol* **35**: 818–822, 1989.
32. Haniu M, Yuan M, Chen S, Iyanagi T, Lee TD and Shively JE, Structure–function relationship of NAD(P)H: quinone reductase: characterisation of NH-2-terminal blocking group and essential tyrosine and lysine residues. *Biochemistry* **27**: 6877–6883, 1988.