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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

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Abstract—Walker tumour cells *in vivo* or *in vitro* are exceptionally sensitive to the monofunctional alkylating agent 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) (Cobb LM *et al.*, *Biochem Pharmacol* **18**: 1519–1527, 1969). CB 1954 forms DNA interstrand crosslinks in a time-dependent manner in Walker tumour cells but not in non-toxically affected Chinese hamster V79 cells [(Roberts JJ *et al.*, *Biochem Biophys Res Commun* **140**: 1073–1078, 1986)]. However, co-culturing Chinese hamster V79 cells with Walker cells in the presence of CB 1954 renders the hamster cells sensitive to CB 1954 and leads to the formation of interstrand crosslinks in their DNA, findings indicative of the formation by Walker cells of a diffusible toxic metabolite of CB 1954. A flavoprotein, of molecular weight 33.5 kDa as estimated by SDS–polyacrylamide gel electrophoresis, has been isolated from Walker cells and identified as a form of NAD(P)H dehydrogenase (quinone) (DT diaphorase, EC 1.6.99.2). This enzyme, in the presence of NADH or NADPH, catalyses the aerobic reduction of CB 1954 to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This new compound can form interstrand crosslinks in the DNA of Chinese hamster V79 cells to which it is also highly toxic.

CB 1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide (**1**, Fig. 1) exhibits a dramatic inhibitory effect on the growth of the Walker rat carcinoma *in vivo* and on the division of Walker carcinoma cells *in vitro* [1, 2]. The mechanism of this selective action of CB 1954 against the Walker carcinoma has been the subject of continuing interest since the initial reports of its activity were published about 20 years ago principally in the hope that a human tumour could be found which shared the Walker tumour's sensitivity (see Refs 3 and 4). In fact CB 1954 has been described as "a drug in search of a human tumour to treat" [4]. This work gave rise to a number of theories on its possible mechanism of action. The compound is a monofunctional alkylating agent by virtue of the single aziridine group. However, the ability of a variety of aminoimidazole carboxamides, anthranilamide and adenine to protect against the cytotoxic effects of CB 1954 led to the theory that it acted as a purine antimetabolite [5]. This theory was supported by the observation that CB 1954 was an inhibitor of ribonucleoside diphosphate reductase [6], an enzyme closely involved in DNA synthesis.

We have recently shown [2] that the selective cytotoxicity of CB 1954 can be accounted for by its

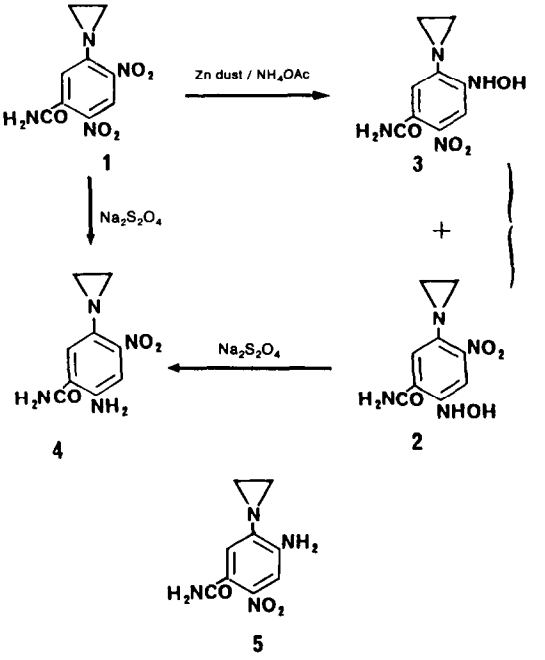

Fig. 1. Formulae of CB 1954 (**1**) and its reduced derivatives: 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (**2**), 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (**3**), 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide (**4**) and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (**5**).

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ability to act as a difunctional agent by forming DNA interstrand crosslinks in Walker, but not other cell lines. Difunctionally-reacting alkylating agents which can produce DNA interstrand crosslinks are known to be potent tumour inhibitors and CB 1954 was claimed to show many properties of these agents both in mammalian cells [7] and bacteria [8]. Such interstrand crosslink formation is impossible for a monofunctional alkylating agent such as CB 1954. However, this compound could possibly be metabolically activated to an agent capable of reacting difunctionally with DNA and thus produce the observed crosslinks.

Initial experiments established that the supernatant obtained after sedimentation of a sonicate of Walker cells, but not of Chinese hamster V79 cells, caused the disappearance of the absorption at 325 nm of CB 1954 [2], strongly suggesting that activation of CB 1954 occurred by metabolism of at least one of its auxochromic nitro groups. This possibility was subsequently supported by the observation that CB 1954 was much less toxic and mutagenic in a nitroreductase-deficient strain of bacteria [9].

We now report the isolation of a pure protein from Walker tumour cells which is able to reduce CB 1954 to a specific hydroxylamine derivative. This novel compound is highly cytotoxic to CB 1954-resistant Chinese hamster V79 cells and can produce interstrand crosslinks in their DNA.

MATERIALS AND METHODS

Cells and labelling conditions. Walker 256 tumour cells were grown in suspension culture in DMEM (Gibco, NY) supplemented with 10% horse serum and 1 mM glutamine [10] and Chinese hamster V79 cells in stirred suspension culture in Eagle's spinner medium [11]. Cells were radiolabelled as previously described [2].

Determination of effects of agents on cell survival. The effects of CB 1954 or its reduction products on the survival of either Walker cells [10] or V79 cells [11] were determined as previously described. To determine the effect of enzyme-activated CB 1954 on the survival of V79 cells, 1 ml volumes of V79 cells (2×10^5/ml) in either growth medium or PBS were treated with 50 μM CB 1954 (5 μl; 10 mM in dimethyl sulphoxide), 500 μM NADH (5 μl; 100 mM in PBS), and 50 μg/ml enzyme (25 μl; 2 mg/ml in PBS), or with the solvent only, as indicated. 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 CB 1954 by HPLC, as detailed later.

Determination of DNA interstrand crosslinking. [^{3}H]Thymidine-labelled Walker or V79 cells were treated with CB 1954 or its reduction products and after various periods of time collected, washed and mixed with control [^{14}C]thymidine-labelled control, untreated, cells for sedimentation in alkaline sucrose, all as previously described [12, 13].

Conditions for co-culture experiments. Replicate 80 cm^2 flasks containing 8×10^6 [^{14}C]thymidine-labelled V79 cells, growing as near confluent monolayers, in 20 ml V79 growth medium were prepared. To these were added 30 ml Walker cell growth medium either containing or not containing, as indicated, 1.6×10^7 [^{3}H]thymidine-labelled Walker cells. These mixed cultures were then treated with various doses of CB 1954 and after 24 hr the two cell types were separated by pouring off the Walker cell-containing supernatant, rinsing the V79 monolayer with PBS (10 ml) and harvesting with trypsin. The V79 cells were then assayed for their survival by colony formation and for the presence of crosslinks in their DNA by alkaline sucrose gradient sedimentation analysis, either alone, or co-sedimented with the [^{3}H]thymidine-labelled Walker cells, as appropriate. Alternatively, Walker cells alone were exposed to CB 1954 for 24 hr, the cells removed from the medium, and the medium added to a culture of V79 cells, all culture conditions being the same as those above. Cells were subjected to 5 Gy gamma irradiation prior to lysis and sedimentation as previously described [12, 13].

Synthesis of 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (2) and 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (3). To a vigorously stirred solution of CB 1954 (compound **1**) (1.008 g) in acetone (40 ml) was added zinc dust (500 mg) and ammonium acetate (500 mg) with repeat additions of these amounts of zinc and ammonium acetate after 2,4 and 6 min. After 8 min, solid was removed by filtration and the orange–red filtrate diluted with CH_2Cl_2 (60 ml) and applied to a column (80 g, 250 × 30 mm) of silica gel (Merck, Art. No. 9385). Elution under pressure (N_2) with acetone/CH_2Cl_2 (1:1, 125 ml pre-run, then 20 ml fractions) gave, respectively, compounds **1** (770 mg, fractions 1–8), **2** (fractions 9–15), a mixture of **2** and **3** (fractions 16–19) and **3** (fractions 20–31) (Fig. 1). Concentration to 5 ml of those fractions containing **2** gave garnet crystals (17 mg, 8% yield, based on unrecovered CB 1954) which darkened without melting above 200°, ^{1}H-NMR (Bruker AC spectrometer, 250 MHz, solution in d_6-DMSO): δ 2.20 (s, 4H, aziridinyl H), 7.42 (s, 1H, H-6), 7.63 (s, 1H, H-3), 7.71 (s, 1H, one of amide NH), 8.23 (s, 1H, other of amide NH), 8.85 (d, 1H, *J* 2.2 Hz, OH), 9.12 (d, 1H, N*H*OH); Anal: Calc. for $C_9H_{10}N_4O_4$: C, 45.4; H, 4.2; N, 23.5. Found: C, 45.1; H, 4.3; N, 23.4%. Concentration to 20 ml of those fractions containing **3** gave minute yellow crystals (29 mg, 13% yield based on unrecovered **1**) which darkened above 200° without melting, NMR: δ 2.14 (s, 4H, aziridinyl H), 6.94 (s, 1H, H-6), 7.43 (s, 1H, H-3), 7.45 (s, 1H, one of amide NH), 7.89 (s, 1H, other of amide NH), 8.57 (brs, 1H, N*H*OH), 8.88 (d, 1H, *J* 1.5 Hz, OH); Anal: Found; C, 45.2; H, 4.1; N, 23.1%.

2-Amino-5-(aziridin-1-yl)-4-nitrobenzamide (compound **4**) and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (compound **5**) were prepared by the published procedure [14]. Their NMR spectra, not previously reported, are recorded here for comparison with those of compounds **2** and **3**; NMR of **4**: δ 2.11 (s, 4H, aziridinyl H), 6.46 (brs, 2H, NH_2), 7.24 (s, 1H, H-6), 7.32 (s, 1H, H-3), 7.46 (brs, 1H, one of amide NH), 8.01 (brs, 1H, other of amide NH). NMR of **5**: δ 2.11 (s, 4H, aziridinyl H), 5.74 (brs, 2H, NH_2), 6.69 (s, 1H, H-6), 7.09 (s, 1H, H-3), 7.31 (brs, 1H, one of amide NH), 7.79 (brs, 1H, other of amide NH).

Isolation of a protein with a nitroreductase activity from Walker tumour cells. The steps involved in the isolation and purification of the protein that activates CB 1954 are illustrated by electrophoresis of the resulting protein(s) through SDS–polyacrylamide (12%) gels and their subsequent location by staining with Coomassie blue [15] as shown in Fig. 2.

Walker 256 cells growing in stirred suspension culture were resuspended at 2×10^8 cells/ml with 1% aprotinin in ice-cold PBS and disrupted by sonication. The pale yellow supernatant (A, Fig. 2) (5 ml), obtained after centrifugation (10,000 *g* for 1 hr at 4°), was injected onto a TSK G3000 SWG (21.5 × 600 mm) gel filtration column, eluted at ambient temperature (3 ml/min) with 0.01 M sodium phosphate buffer (pH 7), and 3 ml fractions collected. Nitroreductase activity in the fractions was detected by the loss of CB 1954 as determined by HPLC: a solution (5 μl) of CB 1954 (300 μM) and NADH (5 mM) in PBS was incubated with fractions from the column (50 μl), the mixture incubated at 37° for 1 hr, then aliquots (10 μl) injected onto an ODS-5S reverse-phase HPLC column and eluted isocratically (1 ml/min) with 0.1 M ammonium acetate (pH 7) in 25% methanol. The eluate was continuously monitored for absorption at 325 nm. CB 1954 eluted as a single resolved peak after about 7 min. Fractions containing reductase activity were pooled and concentrated by centrifugation through a polysulfone membrane (Millipore Ultrafree 30,000, Bedford, MA) and 2 ml of the concentrate (B, Fig. 2) re-injected onto the above GFC column. Active fractions were again pooled, concentrated (C, Fig. 2) and the concentrate (2 ml) injected onto a TSK DEAE-5PW (7.5 × 75 mm) anion exchange column, eluted with a NaCl gradient (0–0.5 M linear over 40 min) in sodium phosphate buffer (pH 7) (1 ml/min) and 0.5 ml fractions collected. Again active fractions were pooled, concentrated (D, Fig. 2) and finally the concentrate (2 ml) re-injected onto the above column. Protein purity (>95%) was established by SDS–polyacrylamide gel electrophoresis, the nitroreductase protein being detected as a single band of 33.5 kDa (E, Fig. 2). A similar molecular weight for this protein was determined by gel filtration chromatography. The protein concentration was determined by its absorbance at 450 nm given that 1 mg/ml gave an absorbance of 0.34 (10 mm pathlength) as determined by a standard protein assay (Bio-Rad) calibrated with bovine serum albumin.

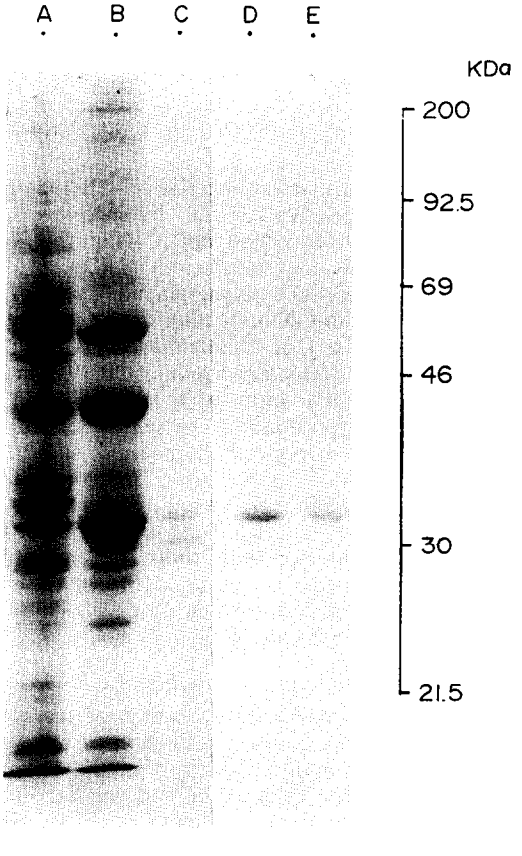

Fig. 2. Isolation of a protein with a nitroreductase activity from Walker tumour cells. The illustration of steps involved in the isolation and purification of the protein that activates CB 1954 (as described in Materials and Methods) by electrophoresis of the resulting protein(s) through SDS–polyacrylamide (12%) gels and their subsequent location by staining with Coomassie blue. (A) crude extract, (B) after the first gel filtration step, (C) second gel filtration, (D) first anion exchange step and (E) after the second anion exchange step.

The enzymic reduction of labelled CB 1954. A mixture of [U-^{3}H]CB 1954 [14] (30 μM; 140 μCi/mMol), NADH (500 μM) and the purified enzyme (50 ng) was incubated in PBS (1 ml) at 37°. At various times a sample (200 μl) was removed and injected onto 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 0.1 M sodium phosphate buffer (pH 7). Samples (1 ml) were collected and the tritium activity of each determined by liquid scintillation counting.

Cytotoxicity of the enzymic reduction products of CB 1954: CB 1954 (200 μM), NADH (5 mM) and enzyme (100 ng) were incubated in PBS (600 μl) at 37°. After 2 hr, an aliquot (500 μl) was injected onto the ODS-5 HPLC column and eluted as described for the enzymic reduction above. Samples (1 ml) were collected, individually sterilised by passage through 0.2 μm filters and a proportion (500 μl) added to a culture of Walker 256 cells (10 ml/2×10^5 per ml) which, after a 2 hr incubation at 37° were assayed for colony-forming ability. The cytotoxicities of the synthesised hydroxylamino derivatives of CB 1954 towards Walker cells were determined by a method similar to the above. A sample (200 μl) of a 200 μM solution of an equal mixture of the 2- and the 4-hydroxylamines was injected onto the ODS-5 HPLC column and the toxicities of the resulting fractions towards Walker cells determined as previously.

RESULTS

Evidence for a cytotoxic metabolite of CB 1954 *in Walker cells*

Initial evidence that the cytotoxic, DNA cross-

linking ability of CB 1954 in Walker cells is effected by a soluble and diffusible agent, probably a metabolite of CB 1954, was obtained by exposing otherwise insensitive Chinese hamster V79 cells to CB 1954 in the presence of Walker cells, the two cell types sharing intimate contact in the same medium. Under these co-culturing conditions, the V79 cells evinced dramatic sensitivity to CB 1954 and crosslinks could be detected in their DNA, Thus, when V79 and Walker cells were co-cultured in the absence of CB 1954 the sedimentation profiles of their respective labelled DNAs were identical (A, Fig. 3) and indicative of Mn values of about 2×10^8 daltons (as expected for the DNA of cells irradiated with 5 Gy). Addition of 3 μM (B, Fig. 3) or 5 μM (C, Fig. 3) CB 1954 to the cell mixture resulted not only in a dose-responsive increase in the amount of Walker cell DNA sedimenting further into the gradient, as seen previously [2] and characteristic of crosslinks, but also, though to a lesser extent, an increase in the proportion of V79 cell DNA sedimenting further into the gradient and similarly indicative of the presence of crosslinks. No such occurrence of crosslinks in the DNA of V79 cells was seen when they were incubated for 24 hr in medium in which Walker cells had been grown in the presence of CB 1954 for the previous 24 hr and then removed (D, Fig. 3).

When V79 cells were treated with CB 1954 in the presence of Walker cells, as above, and assayed for cell survival, there was a dose-dependent decrease in the surviving fractions as compared with no toxic effects of these concentrations of CB 1954 in the absence of Walker cells (Fig. 4).

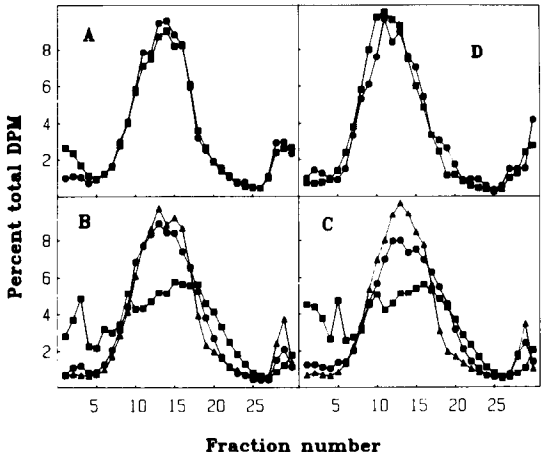

Fig. 3. The effects of co-culturing Walker cells and Chinese hamster V79 cells in the presence of CB 1954 on the sedimentation characteristics of their labelled DNAs. Cell types were mixed or not as detailed, and exposed to 0 μM (A), 3 μM (B), or 5 μM (C) CB 1954 for 24 hr: (●), V79 mixed, (■), Walker mixed, (▲), V79 alone. Additionally, (D), V79 cells were grown for 24 hr in medium previously conditioned by Walker cells plus 10 μM CB 1954 for 24 hr: (●), control, (■), conditioned medium. Cells were harvested, washed with PBS then irradiated (5 Gy) and their DNA analysed by sucrose gradient sedimentation. The direction of sedimentation is from left to right.

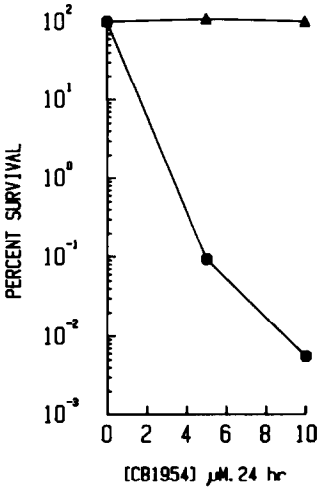

Fig. 4. The effects of co-culturing Walker cells and Chinese hamster V79 cells in the presence of CB 1954 on the survival of the V79 cells. V79 cells were exposed to doses of CB 1954 for 24 hr in medium shared with Walker cells as detailed in the text. The V79 cells were then re-isolated and assayed for colony formation. (▲) Control V79 cells, (●) Co-cultured V79 cells.

Characterisation of a protein with nitroreductase properties

A pure protein that can metabolise CB 1954 has been isolated from Walker cells by successive gel filtration and ion exchange HPLC (Fig. 2). This protein has a molecular weight of 33.5 kDa, as estimated from the SDS–polyacrylamide gels. The characterisation and identification of the protein is reported elsewhere [16]. In summary, it is a FAD-containing protein which can reduce CB 1954 in air or nitrogen at similar rates using either NADH or NADPH as electron donors. The sequence analysis showed almost complete homology with the sequences published for NAD(P)H dehydrogenase (quinone) (DT-diaphorase, menadione reductase, quinone reductase, EC 1.6.99.2) isolated from rat liver [17–19], but not with those of any other sequenced protein [20]. The similarity of the two enzymes has been further indicated by studies of their substrate specificities [16].

Identification of the toxic metabolite of CB 1954

An indication of the likely nature of the toxic product formed by enzymic reduction of CB 1954 came initially from a comparison of the *in vitro* response of Walker cells to the two isomeric nitro amines obtained by partial reduction of CB 1954. As shown in Fig. 5, 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide (compound 4) was appreciably more toxic to Walker cells than 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (compound **5**). This observation strongly indicated the requirement for a reducible nitro group at the 4-position, i.e. ortho to the aziridine group, for the cytotoxic activity of CB 1954 and this was confirmed when products formed by the enzymic reduction of CB 1954 were identified.

Incubation of ^{3}H-labelled CB 1954 with the nitro-

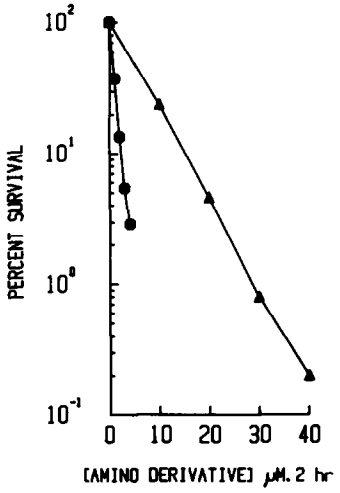

Fig. 5. The effect of 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide (compound **4**) (●) and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (compound **5**) (▲) on the survival of Walker cells. Walker cells were exposed to the two amino derivatives for 2 hr, at 37°, and then assayed for colony forming ability.

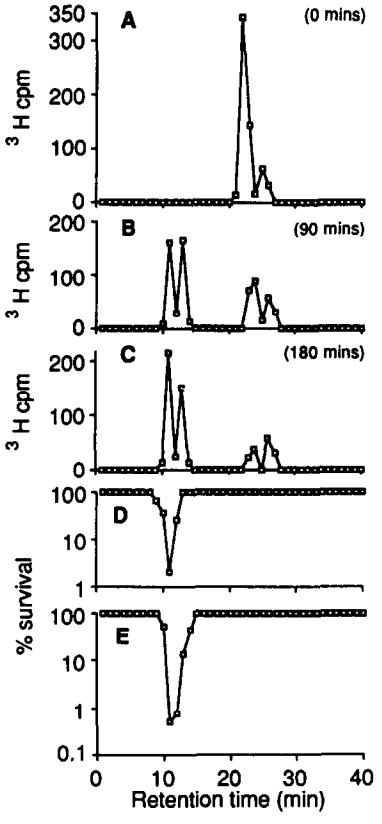

Fig. 6. The products generated by the enzymic reduction of CB 1954 for (A) 0 min; (B) 90 min; (C) 180 min. The resulting cytotoxicity of these products (plotted as surviving cell fraction against elution time from the column) is shown in (D). Similarly the cytotoxicity of a mixture of the 2- and 4-hydroxylamino derivatives of CB 1954 is shown in (E). Elution times of the standards were: CB 1954 (compound **1**) 23.1 min, 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (compound **2**) 27.2 min, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (compound **3**), 10.8 min, 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide (compound **4**), 25.4 min, and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (compound **5**) 13.2 min.

reductase enzyme, purified as above, and analysis of the resulting products by reverse-phase HPLC indicated a time-dependent loss of CB 1954 and the appearance of two new ^{3}H-labelled products eluting at earlier times than did CB 1954 (A–C, Fig. 6). However, only one of these compounds was toxic towards Walker cells at the concentration eluted from the column (D, Fig. 6). The cytotoxic product was shown, by comparison of its elution and spectral characteristics with an authentic sample of the synthetic compound, to be 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (compound **3**) (E, Fig. 6). The other labelled compound was tentatively identified as 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (compound **5**) by its elution characteristics, but as it co-eluted with the NADH no further confirmation was attempted. Thus the enzyme does not readily reduce the nitro group at the 2 position in CB 1954 and the synthetic 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (compound **2**) was shown to be far less toxic towards Walker cells than the above 4-hydroxylamino compound (E, Fig. 6).

Chemical reduction of CB 1954. The method chosen for the unambiguous synthesis of the two possible hydroxylamines formed by partial reduction of CB 1954 was essentially that used to prepare the hydroxylamino analogue of aminoglutethimide, namely reduction of the nitro-derivative using zinc dust and ammonium acetate in acetone [21]. Use of the more usual ammonium chloride was less satisfactory and an attempt to use a more recently described procedure for reducing nitroarenes to arylhydroxylamines using selenium and sodium borohydride in ethanol [22] was thwarted by the poor solubility of CB 1954.

The presence of hydroxylamino functions in the reduction products was shown by NMR spectroscopy. Thus the appropriate OH and NH signals were observed, being distinguishable by the relatively better resolution of the doublet signals for the OH proton and by the slower exchange of the NH proton which was still visible 5 min after addition of 10% D_2O whereas the OH signal was not. However, these spectra do not establish the relative orientation of the hydroxylamino and aziridino functions. To do this, the hydroxylamino derivative of higher R_f value (0.32) in dichloromethane–ethanol (5:1), was reducd on a small scale using dithionite in aqueous acetone [14], conditions which selectively reduced CB 1954 to the known 2-amino-4-nitro derivative **4**. This product was chromatographically identical with compound **4** (R_f, 0.38), establishing the structure **2** for this 2-hydroxylamino derivative. The structure **3** was therefore assigned to the isomer of lower R_f value (0.29) whose structure could not be independently assigned by reduction to the 4-amino-2-nitro derivative **5** as the latter had the same R_f value. Because of this coincidence it was particularly

important to establish that **5** was not a contaminant in the sample of **3**. Fortunately their ^{1}H-NMR spectra permit this: in particular the chemical shift values for H-3 and H-6 are distinct for all four compounds **2–5**.

The yields of **2** and **3** were variable and modest, and much starting material was recovered following column chromatography. Attempts to improve the yield by further additions of the reducing agents caused further reduction to products other than **2** and **3** which also gave blue or blue–purple products when thin-layer chromatograms were sprayed with aqueous sodium pentacyanoammine ferroate, characteristic of the hydroxylamino function [23]. These products were not further characterised. The yields of **2** and **3** were further compromised by their instability when concentrated in organic solvents and their limited solubility in the chromatographic eluant. The consequent need to apply them to the column in dilute solution precluded complete separation of **2** and **3**,

Formation of DNA interstrand cross links by 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide

Final confirmation of the notion that the selective toxicity of CB 1954 to Walker cells was due to the enzymic formation of a specific DNA crosslinking agent and that this was 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (compound **3**), was obtained by demonstrating the ability of this compound to form DNA interstrand crosslinks in, and be toxic towards, Chinese hamster V79 cells. Initially we used the hydroxylamine derivative generated from CB 1954 by means of the above enzyme and, subsequently, the synthetic product. Treatment of Chinese hamster V79 cells with CB 1954 and NADH had no effect on the subsequent sedimentaiton of their labelled DNA. However, inclusion of increasing amounts of the purified enzyme produced a progressive increase in the amount of DNA that was crosslinked (Fig. 7A), as indicated by the increasing proportion of DNA of higher molecular weight which sediments further into the alkaline sucrose gradient. A concomitant decrease in the surviving fraction of the treated cells from 1 in the absence of enzyme to less than 10^{-4} in its presence was seen (Table 1). A minor toxic effect of CB 1954 and NADH alone was seen. Analysis by HPLC of the medium in which the cells had been treated revealed a decrease in the concentration of CB 1954 from 50 μM to about 25 μM in those experiments where both NADH and enzyme were present but no significant decrease if either of these components was omitted (Table 1). No differences in either the reduction of CB 1954 or the resulting cytotoxicity to V79 cells was seen, whether the reactions were performed either in fully complemented tissue-culture medium, or in PBS.

Treatment of Chinese hamster V79 cells with the chemically synthesised 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide also produced DNA interstrand crosslinks, although there was only slightly more crosslinking following treatment with 20 μM than with 5 μM (Fig. 7B). At doses of 50 μM and 100 μM of the 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide there was no further increase in DNA crosslinking, increasing strand-breakage of DNA being seen (data not shown). Less DNA crosslinking was observable with the pure 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide than could be generated by means of the enzymic activation of CB 1954.

When the Chinese hamster V79 cells were assayed for colony forming ability after treatment with 20 μM 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (a dose similar to that presumably generated by the enzymic reduction of CB 1954 above) a surviving fraction of about 10^{-5} was seen (Table 1). The same dose of 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide was much less cytotoxic than the 4-hydroxylamino derivative, and CB 1954 was non-toxic at a similar dose (Table 1).

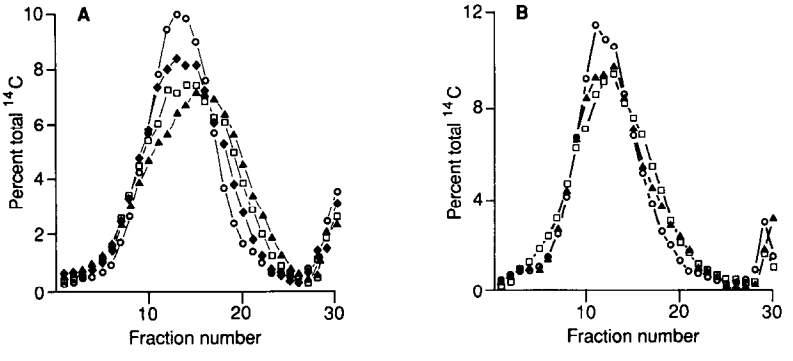

Fig. 7. (A). The formation of crosslinks in the DNA of Chinese hamster V79 cells treated with CB 1954 and the nitroreductase enzyme. The V79 cells were treated with CB 1954 (10 μM) and NADH (100 μM) and various quantitites of the purified enzyme for 18 hr, washed with PBS then irradiated (5 Gy) and their DNA analysed by sucrose gradient sedimentation. (○) no enzyme; (◆ 1 μg/ml; (□) 10 μg/ml; (▲) 100 μg/ml nitroreductase enzyme. The direction of sedimentation is from left to right. (B). The formation of crosslinks in the DNA of Chinese hamster V79 cells treated with 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. V79 cells were treated with various doses of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide for 2 hr, washed with PBS, irradiated (5 Gy) and subjected to alkaline sucrose gradient sedimentation as in 7(A). (○) untreated; (▲) 5 μM; (□) 20 μM 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. The direction of sedimentation is from left to right.

Table 1. The effect of CB 1954 in the presence of the nitroreductase enzyme and NADH, on the survival of Chinese hamster V79 cells

	Treatment protocol					
	1	2	3	4	5	6
CB 1954 (50 μM)	−	+	+	+	−	−
NADH (500 μM)	+	−	+	+	−	−
Enzyme (50 μg/ml)	+	+	−	+	−	−
4-Hydroxylamino-CB 1954 (20 μM)	−	−	−	−	−	+
2-Hydroxylamino-CB 1954 (20 μM)	−	−	−	−	+	−
CB 1954 Concentration after 2 hr (μM)						
(i) Medium	0	50.5	43.3	24.5		
(ii) PBS	0	44.6	51.3	25.4		
Survival (%)						
(i) Medium	100	100	41	<0.01	14.31	0.00115
(ii) PBS	100	100	32	<0.01		

Treatment protocols used: omitting CB 1954 (1), NADH (2), enzyme (3), or nothing (4). Additionally, V79 cells were exposed to only 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide (5), or 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (6). V79 cells were exposed to the agents in either V79 cell growth medium or PBS, for 2 hr at 37° and then assayed for colony-forming ability. The medium in which the cells had been treated was retained and then assayed for CB 1954 content by HPLC.

DISCUSSION

The results presented here further confirm our earlier conclusions that the sensitivity of Walker cells to CB 1954 is a consequence of their ability to metabolise the compound to a difunctional agent capable of introducing interstrand crosslinks into cellular DNA. This metabolite was shown to be soluble and diffusible by its ability to be both cytotoxic towards and to crosslink DNA in, CB 1954-resistant V79 cells when they were co-cultured with Walker cells. These effects were not seen when V79 cells were incubated in medium pre-conditioned for 24 hr by Walker cells and CB 1954, indicating either a requirement for intimate cellular contact, or a relatively short half-life of this active metabolite.

Furthermore these present findings established the presence in Walker cells of an enzyme which can reduce CB 1954 to only one major cytotoxic compound, namely 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide formed by partial reduction of the nitro group ortho (i.e. adjacent) to the aziridine group. An amino derivative, 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide was also tentatively identified as another product of this reduction but this amino compound was shown to be far less cytotoxic than the 4-hydroxylamino derivative. No other reduction products were detected by the use of radiolabelled drug and in particular there was no evidence for the reduction of CB 1954 at the 2-nitro position by the Walker nitroreductase enzyme. Reduction of CB 1954 proceeded at about equal rates under air or nitrogen, and therefore the reaction is not inhibited by oxygen. The only coenzyme required was either NADH or NADPH. Previous studies demonstrated the presence of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide as the primary metabolite in the urine of CB 1954-treated rats. 2-Amino-5-(aziridin-1-yl)-4-nitrobenzamide was not found [14]. In this earlier study, 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide had a potent effect on the Walker tumour *in vivo*, but was also very toxic to the whole animal, resulting in a poor therapeutic ratio. This made 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide a poor antitumour agent in contrast to the less active, but also less toxic 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide [14].

The nitroreductase enzyme isolated from Walker cells that specifically reduces CB 1954 to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide has been identified. The enzyme is a form of NAD(P)H dehydrogenase (quinone) (quinone reductase, EC 1.6.99.2). It has also been called menadione oxidoreductase or DT-diaphorase and the identification and properties of this enzyme, as isolated from Walker tumour cells, is the subject of a further publication [16]. NAD(P)H dehydrogenase (quinone) is an inducible flavoenzyme which catalyses the 2-electron reduction of quinones to hydroquinones and thus has an important role in their detoxification (for a review see Ref. 26). The fact that such an enzyme can also activate CB 1954 to a cytotoxic species by a 4-electron reduction is thus of great interest.

The formation of a second reactive site in the otherwise monofunctional CB 1954 by partial reduction of the nitro group adjacent to the aziridine group is, in retrospect, consistent with the reported activities against the Walker tumour of a large series of related compounds. For example, 1-(aziridin-1-yl)-2-cyano-4-nitrobenzene was inactive while 1-(aziridin-1-yl)-4-cyano-2-nitrobenzene did possess antitumour activity [24, 25].

The enzyme was capable of activating CB 1954 extracellularly in the presence of NADH, to a cytotoxic species, presumably the 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. A slight toxic effect of the combination of CB 1954 and NADH was observed in the absence of the enzyme, perhaps

indicating a small degree of non-enzymic reaction between these two compounds, or stimulation by the NADH of a small amount of CB 1954 reduction by the V79 cells. The toxicity observed in V79 cells when they were exposed to chemically-synthesised 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide was similar to that of an equivalent dose of the hydroxylamine produced by enzymic reduction of CB 1954, and these respective treatments produced similar degrees of DNA interstrand crosslinking. It is of interest to note that the enzyme does not reduce the nitro group at the 2-position of CB 1954, and moreover, that the chemically synthesised product 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide was much less cytotoxic towards both Walker and V79 cells, than 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide.

At higher doses of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to V79 cells, there was no further increase in DNA crosslinking, progressive DNA strand breakage being observed. This phenomenon is reminiscent of that seen when Walker cells were treated with increasing doses of CB 1954 for a 2 hr period (data not shown). Here again, a dose-responsive formation of DNA interstrand crosslinks was only seen over a limited range, above which progressive strand breakage was seen. The observed DNA strand breakage was presumably the result of monofunctional DNA reactions of the agent, although the alkaline environment of the sedimentation assay did not permit distinction between alkali-labile sites and frank breaks. These monofunctional reactions, being purely chemical, should accumulate in a dose-responsive manner. By contrast, the crosslinking reactions of CB 1954 are dependent upon its enzymic reduction, and this requirement for activation could explain the limited dose-responsiveness of DNA crosslinking. However, this lack of a dose response in crosslinking is seen not only in Walker cells treated with CB 1954, but also in V79 cells treated with the active metabolite. This suggest that in cells there is further activation involving a metabolic step that converts the 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to the proximal, DNA-crosslinking, cytotoxic species. Presumably the observed strand breakage is the result of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide continuing to react monofunctionally, when at higher doses the above metabolic conversion is saturated. This notion is corroborated by the observation that 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide produced no apparent crosslinking in naked DNA, and strand breakage was seen (data not shown). The activation probably involves the metabolic esterification of the 4-hydroxylamino group in a manner analogous to that found for the activation of the hydroxylamines formed by metabolism of 4-nitroquinoline-*N*-oxide [27] and *N*-acetylaminofluorene [28].

Whilst DNA interstrand crosslinks are formed by the bioactivation of CB 1954 they are almost certainly not the only lesions introduced into cellular DNA. Other lesions would be expected to include both monofunctional adducts and other difunctional adducts such as intrastrand crosslinks between the various bases on the same strand of DNA. However, it is well established that agents capable of forming DNA interstrand crosslinks are potent cytotoxic agents and are useful in the chemotherapy of human cancer, although the quantitative effect of the interstrand crosslink in producing cytotoxicity will depend on the phenotype of a given cell (for a full discussion see [13]).

In summary, our findings adequately explain the sensitivity of Walker tumour cells to CB 1954 as a consequence of the selective reduction of this compound to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a molecule which in cells can crosslink DNA. A single enzyme can catalyse this reduction, and this enzyme has been shown to be a form of NAD(P)H dehydrogenase (quinone).

These observations could lead to the development of new anti-cancer strategies. Antibodies to the enzyme could lead to the detection of CB 1954-sensitive human tumours, while new anti-cancer drugs could be developed from the defined active metabolite of CB 1954. We have shown that the enzyme is capable of reducing CB 1954 to its cytotoxic species under physiological conditions. Thus the targeting of the nitroreductase enzyme to a tumour cell by means of a coupled tumour-specific antibody could result in the activation of the drug only at the site of a tumour and thereby provide a novel approach to antitumour chemotherapy [29]. Finally, it is indeed possible that CB 1954 may have a role in the chemotherapy of human tumours now that the basis of its selective action towards Walker cells is understood.

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