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IDENTIFICATION, SYNTHESIS AND PROPERTIES OF 5-(AZIRIDIN-1-YL)-2-NITRO-4-NITROSOBENZAMIDE, A NOVEL DNA CROSSLINKING AGENT DERIVED FROM CB1954

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Abstract—5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, the active form of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954), can react spontaneously with oxygen, and in aqueous solution yields 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide and hydrogen peroxide. Mild biological reducing agents such as NAD(P)H, reduced thiols and ascorbic acid rapidly re-reduced the nitroso compound to the hydroxylamine. Both compounds were equally efficient at inducing cytotoxicity and DNA interstrand crosslinking in cells when exposed in phosphate-buffered saline (PBS). Neither agent was capable of inducing cross-links in isolated DNA. When acetyl coenzyme A was included in the incubation, crosslink formation was seen with the hydroxylamine, but not with the nitroso compound. Thus, the nitroso compound is acting as a prodrug for the hydroxylamine, and needs to be reduced to this compound to exert its cytotoxic effects. *In vivo* anti-tumour tests showed that neither compound was effective in its own right. This may be due to the rapid reduction of the nitroso to the hydroxylamine, and the reaction of the hydroxylamine with serum proteins. The chemical synthesis of the 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide, and an improved synthesis of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is described. These results emphasize the potential efficacy of the *in situ* activation of prodrugs such as CB1954 either by endogenous enzymes such as DT diaphorase, or by antibody directed enzyme prodrug therapy (ADEPT).

5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB1954‖) (**1**, Fig. 1) can be an exceptionally effective antitumour agent. In the rat Walker 256 tumour, CB1954 has a much greater therapeutic index than successful anticancer drugs such as chlorambucil and cisplatin [1]. Such activity was not expected from a monofunctional alkylating agent such as CB1954. It is now known that CB1954 is a prodrug which is bioactivated by the enzyme DT diaphorase to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (**2**, Fig. 1) a difunctional DNA-crosslinking agent [2–4]. On a dose basis, the increase in cytotoxicity (observed in cells able to bioactivate CB1954) is about 100,000-fold (when compared to cells unable to perform this conversion) [2]. The dramatic tumour selectivity is due to the level of DT diaphorase which is elevated in certain tumours [5, 6] but appears to be low in those normal tissues which are usually sensitive to cytotoxics [7]. In all types of cells, including those insensitive to CB1954, **2** is further activated by non-enzymic reaction with thioesters [8]. It is believed that this reaction produces 4-(*N*-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide, which is the ultimate difunctional species capable of reacting with DNA to produce crosslinks and cytotoxicity [8]. The crosslinks that are induced by CB1954 account for a very high percentage of the total DNA adducts and are, moreover, comparatively resistant to repair [9]. Human tumour cells can contain similar levels of DT diaphorase to CB1954-sensitive rat cells, and human DT diaphorase also reduces CB1954 to **2** [10]. However human cells are insensitive to CB1954. This is because the human enzyme reduces CB1954 about seven times slower than the rat enzyme. Therefore CB1954 is not a useful antitumour agent in humans.

Human DT diaphorase remains in principle an exploitable target for antitumour selectivity (for a recent review see Riley and Workman [11]). This could still apply to CB1954. Reduced nicotinamide nucleosides amplify the activity of human DT diaphorase towards CB1954, and so increase its cytotoxicity [7]. Also, it may be possible to design analogues of CB1954 that are more readily reduced by the human enzyme. Alternatively, CB1954 and its analogues might be prodrugs in an antibody directed enzyme prodrug therapy (ADEPT) [12]. A candidate ADEPT enzyme is an *E. coli* nitroreductase which can bioactivate CB1954 90 times faster than

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‖ Abbreviations: CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; ADEPT, antibody directed enzyme prodrug therapy; PBS, phosphate-buffered saline; DMSO, dimethylsulphoxide.

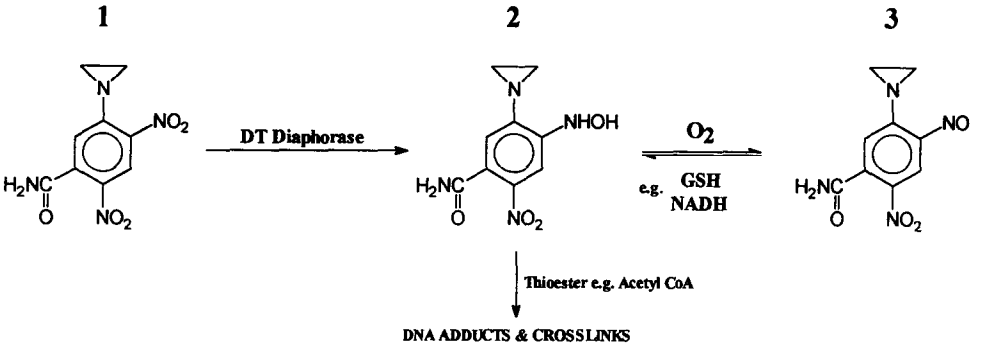

Fig. 1. Formulae and bioactivation of CB1954 (**1**) and its reduced derivatives 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (**2**) and 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (**3**).

DT diaphorase [13]. This enzyme can (unlike DT diaphorase) reduce either of the nitro groups of CB1954 [14] although the 2-hydroxylamine is less cytotoxic. These approaches might recreate in humans the remarkable antitumour efficacy which CB1954 had in the rat.

We therefore wish to understand fully the bioactivation of CB1954. As part of this ongoing study we now report the chemical and biological properties of 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (**3**, Fig. 1), a spontaneous oxidation product of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide.

MATERIALS AND METHODS

Materials. All chemicals and reagents were supplied by the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated.

Cells and growth conditions. Walker 256 tumour cells were grown in Dulbecco's modified Eagle's medium with 10% horse serum and 1 mM extra glutamine. V79 Chinese hamster cells were grown in minimum essential medium with 10% foetal calf serum.

Cytotoxicity assays by colony forming assay. The effects of **2** or **3** on the colony forming abilities of both V79 and Walker tumour cells were assayed as described previously [3]. To measure the cytotoxicity induced by the spontaneous oxidation product of **2** after chromatographic separation, a 1-mL volume of 250 μM **2** was incubated in phosphate-buffered saline (PBS) at 37° for 30 min. A 500-μL volume was then injected onto an ODS-5 HPLC column (Bio-Rad 250 × 4.5 mm) and eluted (1 mL/min) with a methanol gradient (0–30% over 30 min, 30–100% over 10 min) in 10 mM phosphate buffer, pH 7. Fractions (40 × 1 mL) of the eluate were collected, individually sterilized, and 500 μL added to 10 mL Walker cells. After 2 hr, the colony forming ability of the cells were assayed as before [3]. The percentage of cells surviving was plotted against retention time of each fraction. Also plotted was the 340 nm absorbance from a similar experiment, in which only 10 μL was injected onto the HPLC column.

Chemistry: general experimental. ^{1}H-NMR spectra were determined with a Bruker AC250 spectrometer and chemical shifts (δ) are given relative to tetramethyl-silane. Electron impact mass spectra were determined on a VG 7070H spectrometer operating at an ionizing voltage of 70 eV with an ion source temperature of 200°. Thin-layer chromatograms were run on fluorescent silica (Merck 5735) with location of components by UV light and by aqueous sodium pentacyanoammine ferroate (nitroso and hydroxylamine compounds) [15]. Preparative HPLC was carried out on Merck Kieselgel 60 (Art 15111) using an Axxial Chromatospac Prep 10 coupled to a Cecil 212A UV monitor operating at 254 nm. Dry tetrahydrofuran was purchased from the Aldrich Chemical Co. (Gillingham, U.K.).

Synthesis of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (**2**) *from CB1954 using hydrazine hydrate/Pd-C.* To a solution of **1** (1.0 g) in tetrahydrofuran (80 mL) in an ultrasonic bath cooled with ice was added 5% palladium on carbon (0.2 g). Hydrazine hydrate (0.80 mL) was added after 5 min and after a further 15 min the mixture was filtered through Celite and washed through with tetrahydrofuran (caution: ignition can occur at this stage). The filtrate was concentrated to 10 mL, diluted with ethyl acetate (100 mL) then subjected to preparative HPLC (220 g silica) with ethyl acetate-acetonitrile (19:1) as eluate. The fractions containing the product were concentrated to 100 mL, giving 0.265 g (28%) of pale yellow crystals identical to an authentic sample [3].

Synthesis of 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (**3**) *from the 4-hydroxylamino derivative.* (i) Using *N*-acetylimidazole. To a solution of **2** (67 mg), in dimethyl formamide (0.5 mL) was added *N*-acetylimidazole (30 mg). After 30 min, the mixture was partitioned between ethyl acetate (20 mL) and water (10 mL). The concentrated organic phase was chromatographed on a column of silica gel (Merck 9385) (40 g, 14 × 3 cm) with ethyl acetate as eluant. Compound **3** eluted in the first 60 mL and on concentration of the eluate and cooling to −5° gave yellow crystals (3.5 mg, 5%); m.p. > 300°; mass spectrum: *m/z* 236 (M^+; 100%), 219 (22%), 208 (8%), 192 (43%); Anal: Calc. for $C_9H_8N_4O_4$: C,

45.77; H, 3.41; N, 23.72. Found: C, 45.28; H, 3.54; N, 23.46%.

(ii) Using silver carbonate/celite. To a stirred solution of **2** (50 mg) in dry tetrahydrofuran (7.5 mL) silver carbonate/celite (0.20 g) was added [16]. After 2 hr, solids were remove by filtration and a solution of the concentrated filtrate in ethyl acetate (25 mL) was subjected to HPLC (200 g silica) with this solvent as eluant. Concentration to 5 mL and cooling to −20° yielded **3** (33 mg, 65%), identical to material prepared as in (i) above; ^{1}H-NMR [d_6-dimethylsulphoxide (DMSO)]: δ 2.80 (s, 4H, aziridinyl H), 7.05 (s, 1H, arom H), 7.63 (s, 1H, arom H), 7.77, 8.14 (2 × br s, each 1H, amide NH).

Assessment of rate of loss of 5-(aziridin-1-yl)-4-hydrolxylamino-2-nitrobenzamide (**2**) *under anaerobic and aerobic conditions.* Buffer (5 mL; 10 mM phosphate; pH 7; 37°) was de-gassed by sparging with helium. To this was added **2** to a concentration of 100 μM (50 μL of 10 mM solution in DMSO). Samples were withdrawn at intervals and analysed for concentrations of **2** by HPLC (Partisil SCX column (Whatman, 150 × 4.5 mm), eluted isocratically with 10 mM phosphate pH 5, 2 mL/min, monitored by absorbance at 340 nm). After 100 min, the helium sparging was stopped, and the concentration of **2** monitored at intervals as above.

Stoichiometry experiments. Oxygen consumption by **2** was measured using a Clark electrode dissolved oxygen monitor (Yellow Springs Instruments model 5300). Phosphate buffer (5 mL; 10 mM; pH 7) was equilibrated at 37°, and the reaction initiated by addition of 5–50 μL of 100 mM **2** in DMSO. After 10 min 100 μL were withdrawn, and assayed for the content of **3** by HPLC as above. The amount of oxygen consumed was plotted against the amount of **3** formed. After the 10 min, 50 μL catalase (10 mg/mL: 2600 U/mg) was added to the highest dose.

To measure the stoichiometry of reaction of NADH with **3**, 1 mL volumes of 500 μM NADH solution (10 mM phosphate buffer pH 7; room temperature) were treated with volumes up to 325 μL of 10 mM solution of **3** in DMSO. The solutions were immediately assayed for NADH content (**3** being instantly and completely converted to **2**) by HPLC as above. The amount of NADH consumed was plotted against the amount of **3** added. A similar HPLC protocol was used to monitor the reduction of **3** (100 μM), by equimolar amounts of reduced glutathione, cysteine or ascorbic acid.

Preparation of radiolabelled DNA. Cellular DNA was radiolabelled by growth of V79 cells for 24 hr in the presence of [2-^{14}C]thymidine (50 μCi/mmol; 0.1 μCi/mL) (Amersham) followed by a 2-hr label free chase period as described previously [17]. DNA was extracted and purified, again as described previously [17].

Determination of DNA interstrand crosslinking in V79 cells. [^{14}C]Thymidine-labelled (as above) V79 cells were treated with doses of **2** or **3** as indicated for 2 hr, harvested, washed with PBS and mixed with ^{3}H-labelled control cells. Cell suspensions were gamma irradiated (6 Gy) and analysed by alkaline elution as described previously [18, 19].

Determination of DNA crosslinking and strand breaks in isolated DNA. ^{14}C-Labelled DNA, obtained as above, was washed and dissolved in 10 mM sodium phosphate buffer (pH 7) to a concentration of 100 μg/mL (50,000 dpm/mL). The DNA was incubated at 37° for 4 hr in the presence of **2** or **3** (250 μM) in the presence or absence of acetyl coenzyme A (1 mM). Any resulting DNA crosslinking or strand breakage was analysed by sedimentation in alkaline sucrose density gradients, all as described previously [19].

Assessment of the in vitro *anti-tumour properties of 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide* (**3**) *and 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide* (**2**). Animals were maintained on SDS Expanded Rodent diet and water *ad lib.* The ADJ/PC6 plasmacytoma was transplanted into femal Balb C$^-$ mice (weighing 20–25 g) by subcutaneous implantation of a 1 mm^3 fragment. Twenty days post implantation, mice carrying tumours of comparable size were randomized into groups of three mice per dose level, including 10 untreated controls. CB1954, **2** and **3**, were given by daily i.p. injections for 5 days in 10% dimethylacetamide/arachis oil starting on day 20. CB1954 and **3** were given at 3.25, 6.25, 12.5, 25, 50 and 100 mg/kg, and **2** at 1.56, 3.125, 6.25, 25 and 50 mg/kg. The experiments were terminated on day 30 when the tumour weights of control and treated groups were compared. The biological criterion of activity is defined in terms of the therapeutic index (TI) which is the ratio of the dose required to kill 50% of the animals (LD_{50}) to the dose required to reduce the tumour mass by 90% (ED_{90}).

RESULTS

Chemical reduction of CB1954 (**1**) *and oxidation of the resultant hydroxylamine to nitroso-derivatives*

In the previously reported synthesis of **2** [3] only a small percentage of the starting **1** reacted giving a low yield of **2** (13%, based on unreacted **1**). An alternative method of reduction was therefore explored, namely the use of hydrazine in the presence of palladium/carbon (cf. Westra [20]). This could give an improved yield of **2** (28%) although the yield was erratic.

The 4-nitroso compound (**3**) was first obtained serendipitously during attempts to obtain 4-(*N*-acetoxy)-5-aziridin-1-yl-2-nitrobenzamide by acetylation of **2** using *N*-acetylimidazole. This agent can *O*-acetylate hydroxylamine itself [21] and does not liberate acid that could cleave the aziridine residue of **2**. No acetoxy derivative were obtained, and only a poor (5%) yield of a product having the molecular mass and elemental analysis of the nitroso derivative **3**. This compound was subsequently prepared by oxidizing **2** using silver carbonate/celite (cf. Maassen and Boer [16]).

Identification of 5-(aziridin-1-yl)-2-nitro-4-nitroso-benzamide (**3**)

It was initially observed that when an aqueous buffered solution of **2** was left to stand, a conversion to a novel compound occurred. After 30-min aerobic incubation a 20% conversion to this new compound had occurred, and its retention time as indicated by 340 nm absorbance coincided with a new peak of

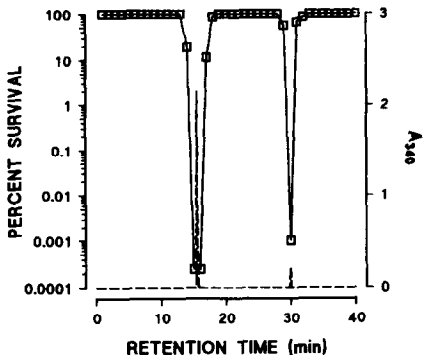

Fig. 2. Co-identification of 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (3) formed by spontaneous oxidation of 5-(aziridin-1-yl)-4-hyroxylamino-2-nitrobenzamide (2) with its cytotoxicity. A solution of 2 was incubated in air at 37° in phosphate buffer pH 7. After 30 min, the resultant mixture was fractionated by HPLC. The cytotoxicity of each fraction towards Walker tumour cells (left ordinate) was assayed by colony forming ability (□). The 340 nm absorbance profile (---) is also shown (right ordinate). Experimental details are described in the text.

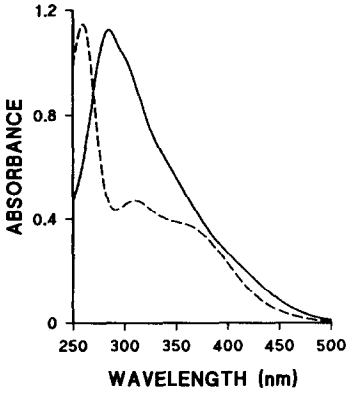

Fig. 3. The spectra of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2) (---) and 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (3) (——). Solutions (100 μM) of the two compounds were prepared in 10 mM phosphate buffer pH 5 and their spectra determined against the appropriate blank (pathlength 1 cm).

cytotoxicity (Fig. 2). Neither the new peak of 340 nm absorbance nor the new peak of cytotoxicity were apparent in the freshly prepared material, the new compound was more hydrophobic than its parent and had a different UV spectrum (Fig. 3). Once formed, the new compound was stable under these conditions (data not shown). It was tentatively identified as 3 and this was confirmed by comparison of elution and spectral characteristics with a synthetic standard. The standard was synthesized and unequivocally identified by NMR, mass spectrometry and elemental analysis as detailed in Materials and Methods. The oxidation of 2 to 3 was shown to be

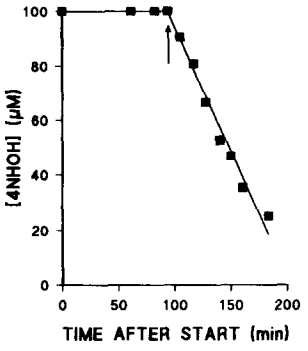

Fig. 4. The stability of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2) under aerobic or anaerobic conditions. A 100 μM aqueous solution (10 mM phosphate; pH 7; 37°) maintained under helium, was periodically assayed for 2 content by HPLC. At the point as indicated by the arrow, the helium atmosphere was replaced by air.

dependent on atmospheric oxygen. When air was excluded, 2 was stable in aqueous solution, but was rapidly oxidized to 3 when the solution was allowed to aerate (Fig. 4). Confirmation of the involvement of oxygen was obtained using an oxygen monitor. Solutions of 2 were incubated in the cell of a dissolved oxygen monitor and the amount of oxygen consumed plotted against the amount of 3 produced. A straight line of slope 0.72 was seen (Fig. 5A). Ten minutes after addition of the highest dose of 2 (1 mM) a 27% reduction in oxygen saturation was seen. Upon addition of catalase, this value returned to 13%. This was taken to indicate a 50% recovery of the oxygen that had been consumed.

When 3 (100 μM) was incubated with the biological reducing agents NAD(P)H, reduced glutathione, cysteine and ascorbic acid, an immediate back-conversion to 2 was observed (data not shown). In the case of NADH it was shown that the only other product was NAD^+. The amount of NADH oxidized was plotted against the amount of 3 reduced, giving a straight line of slope 0.94 (Fig. 5B). The above slopes were taken to indicate, within experimental error, 1:1 stoichiometries.

Cytotoxic and DNA crosslinking properties of 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (3) and 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2) in V79 cells

The chemically synthesized compounds 2 and 3 were seen to have identical cytotoxicities against Chinese hamster V79 cells (Fig. 6A) when treated in PBS. When the cells were treated with the two compounds in fully complemented tissue culture medium (as is the common practice), approximately 10-fold more 3 was required to produce the same cytotoxicity (Fig. 6B). An even greater sparing effect was seen in the case of 2. The two compounds were equally capable of inducing interstrand crosslinks in the DNA of V79 cells (Fig. 7), producing 12.1 crosslinks/10^9 Da of DNA/μM × 2 hr drug treatment.

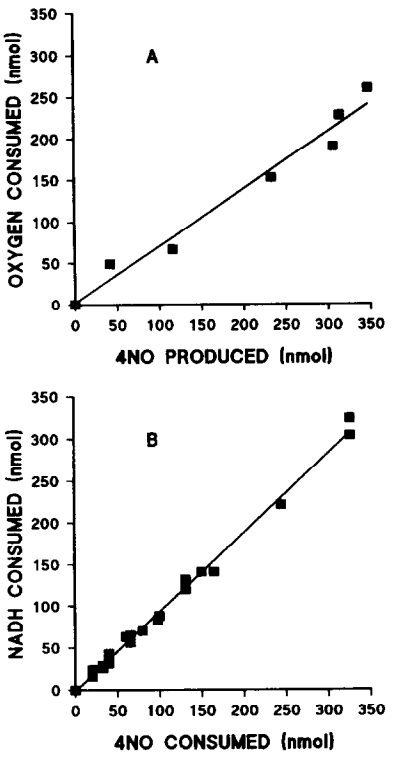

Fig. 5. (A) The stoichiometry of the oxidation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2). Slope of the line = 1.4; $r = 0.9858$. (B) The stoichiometry of the reduction of 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (3) by NADH. Slope of the line = 0.94; $r = 0.9969$. Experimental details are as in the text.

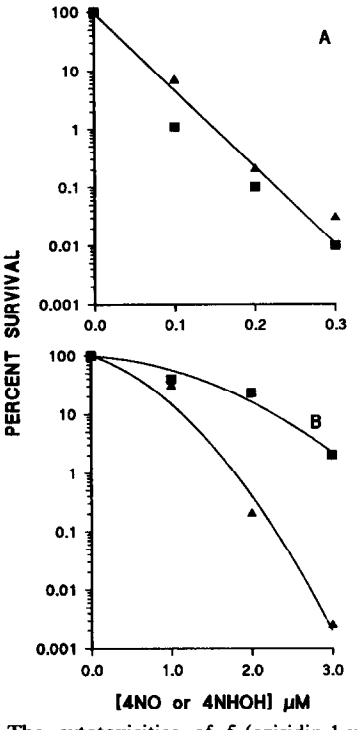

Fig. 6. The cytotoxicities of 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (3) (▲) or 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2) (■) towards V79 cells treated in (A) PBS or (B) tissue culture medium. Cells were treated for 2 hr at 37° and then assayed for colony forming ability.

Crosslinking properties of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2), *and 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide* (3) *towards isolated DNA*

Incubation of ^{14}C-labelled DNA with 250 μM **2** produced a small amount of strand breakage (17.3/10^9 Da of DNA) (Fig. 8A). Inclusion of 1 mM acetyl coenzyme A produced DNA crosslinking (15.8/10^9 Da of DNA), similar to that reported before [8]. By contrast no strand breakage was seen upon incubation with 250 μM **3** (Fig. 8B), and neither was there any evidence of crosslinking when acetyl coenzyme A was included, although a small amount of strand breakage was seen (3.6/10^9 Da of DNA).

Antitumour properties of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (3), *and 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide* (3) in vivo

Compound **2** had an LD_{50} of 70 mg/kg and an ED_{90} of 24.5 mg/kg. As a ratio these give a therapeutic index of 3.4. Compound **3** produced an ED_{90} of 96 mg/kg. At 100 mg/kg no animals died, but a 10% weight loss was evident at the time of death, and it was judged that no animals would survive at 200 mg/kg. Thus the therapeutic index may be stated to be less than 2. Both these therapeutic indices are minimal compared to the high activity of many difunctional alkylating agents in this system. For instance, aniline mustard exhibits a therapeutic index of 80 [1]. Thus, no worthwhile anti-tumour effect was seen. CB1954 has previously been shown to be

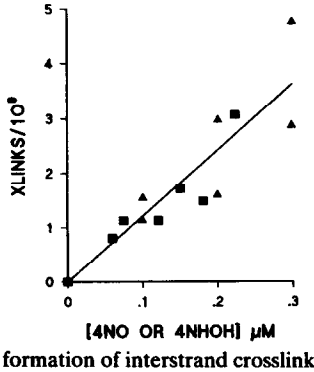

Fig. 7. The formation of interstrand crosslinks in the DNA of V79 cells by 5-(aziridin-1-yl-2-nitro-4-nitrosobenzamide (3) (▲) or 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2) (■). Cells were treated for 2 hr at 37° and crosslink formation was then determined by alkaline elution as detailed in the text.

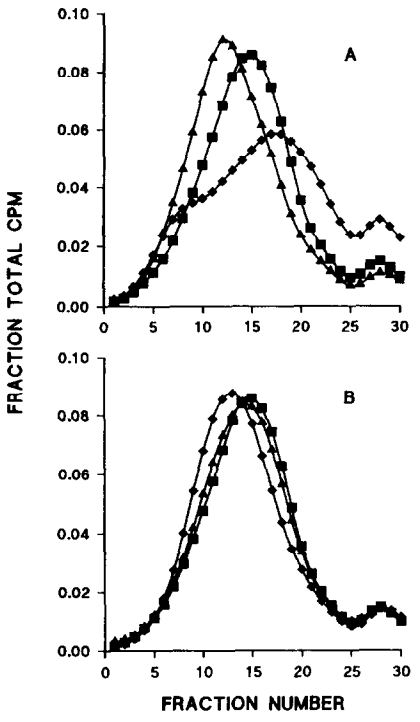

Fig. 8. The formation of crosslinks or breaks in isolated DNA by (A) 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (3) or (B) 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2) and acetyl coenzyme A. The DNA was treated with 2 or 3 (250 μM) with or without acetyl coenzyme A (1 mM) for 4 hr at 37°, and then analysed by sedimentation in alkaline sucrose density gradients. Control (■), agent alone (▲), agent plus acetyl coenzyme A (◆). The direction of sedimentation is from left to right. The crosslink and break frequencies are given in the text.

inactive in this system [22], probably due to low levels of DT diaphorase in this tumour.

DISCUSSION

5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (2) is the product of the reduction by DT diaphorase of CB1954 (1). We now report that under normal aerobic (but not anaerobic) conditions, this compound readily oxidizes to 5-(aziridin-1-yl)-2-nitro-4-nitrosobenzamide (3), and that this process consumes oxygen (Fig. 1). Since this reaction consumes one molecule of oxygen for each molecule of 2 oxidized, the empirical equation would suggest formation of 3 plus hydrogen peroxide.

$$ArNHOH + O_2 \rightarrow ArNO + H_2O_2.$$

This was confirmed by addition of catalase to the reaction mixture, which regenerated half the oxygen that had been consumed. Catalase produces oxygen only from hydrogen peroxide with a stoichiometry of two hydrogen peroxides forming one oxygen and one water molecule. Hydrogen peroxide is a potentially cytotoxic molecule. If it is not destroyed by catalase or peroxidase it can (through interaction with ferrous iron) produce hydroxyl free radicals that react avidly with DNA. Since 3 is re-reduced by NADH (and other biological reducing agents) to 2, the net result is generation of one hydrogen peroxide for each NADH consumed. This redox cycle will continue for as long as oxygen and NADH are present. In a living cell this cycling could continue indefinitely, or until 2 had reacted with other components. This could well explain the DNA strand breakage that is produced in cells seen at higher doses of 2 [3], and in isolated DNA in this paper, and previously [8].

It is shown here that 3 is just as effective as 2 at inducing DNA interstrand crosslinks in cells, but that it has no reactivity towards isolated DNA at all. However, 3 (unlike 2) does not react with acetyl coenzyme A. It may be inferred from this that within the cell 3 is readily reduced back to 2 and that the second-stage activation (by reaction with thioesters) becomes the rate-limiting factor. Thus 3 is a prodrug for 2 and it is 2 that is responsible for the cytotoxic effect (Fig. 1).

Being a potent difunctional DNA crosslinking agent (like chlorambucil, melphalan and cisplatin) and in addition producing crosslinks of unusual properties, 2 might have possessed a useful antitumour activity. Unfortunately its therapeutic index in the ADJ/PC6A system was far too small to indicate usefulness. This is probably because of its high reactivity with serum proteins, and this was reflected in the protection we saw when cells were treated in tissue-culture medium as opposed to PBS. Further, we have previously shown that 2 can diffuse between cells, only when they are in intimate contact [3], implying a very short biological half life. Thus the 2 is probably not able to reach the tumour before becoming deactivated. In itself 3 is a relatively inert compound, but is capable of acting as a prodrug for 2. Therefore, 3 might have been sufficiently durable in the bloodstream to get to a tumour, where it could be converted to 2. In addition, 3 is activated by thiols, whilst resistance to anti-cancer agents such as alkylating agents and cisplatin can be associated with elevated levels of thiols. It was conceivable that 3 could exploit this drug resistance mechanism in its activation to 2. Unfortunately 3 did not prove to be an effective antitumour agent in the ADJ/PC6A system. The reason for this is reflected in the ease of the re-reduction of 3 by other ubiquitous reducing agents such as ascorbic acid. Therefore 3 is probably being too readily reduced in the blood to 2, which as before is rapidly deactivated.

These factors emphasize the potential value of the *in situ* activation of CB1954-like compounds either by DT diaphorase or by a targeted enzyme (ADEPT). Not only is the toxic agent produced selectively at the desired site, but it is unlikely to diffuse far to other sites.

The re-reduction of 3 to form 2 by NADH also raises a mechanistic question about the true product of the reduction of CB1954 by DT diaphorase. It may be that DT diaphorase performs the two-electron reduction of CB1954 to 3, and that the further two-electron reduction to 2 occurs through a purely chemical reaction with NADH. This would be consistent with the usual view of the DT

diaphorase acting through a simultaneous two-electron transfer mechanism. However since the reduction cannot be performed in the absence of NADH, the question cannot be addressed directly, and **2** remains the observed product of the reduction. Further, **3** cannot directly induce the observed cytotoxicity unless it is reduced to **2**. Therefore it remains the case that **2** is the active form of **1**, fully responsible for its DNA crosslinking ability.

In summary, we have extended our previous study of the bioactivation of CB1954 to include a side-reaction of **2** by spontaneous oxidation to yield **3**. The ready re-reduction of **3** to **2** by NADH may contribute some additional toxicity, through generation of hydrogen peroxide. These results emphasize the potential value of prodrugs (such as CB1954) through *in situ* activation either by endogenous enzymes such as DT diaphorase, or by antibody targeted enzymes (ADEPT).

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