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BIOACTIVATION OF CB 1954: REACTION OF THE ACTIVE 4-HYDROXYLAMINO DERIVATIVE WITH THIOESTERS TO FORM THE ULTIMATE DNA–DNA INTERSTRAND CROSSLINKING SPECIES

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Abstract—5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is the active form of CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). This hydroxylamine is formed by the bioreduction of CB 1954 by the enzyme DT diaphorase and accounts for the highly selective cytotoxicity of this compound. The reason why the hydroxylamine derivative is so cytotoxic is that, in contrast to CB 1954, it can react difunctionally as characterized by the formation of DNA–DNA interstrand crosslinks in cells treated by this agent. However, although the 4-hydroxylamine compound can produce these crosslinks in cells it cannot crosslink naked DNA (Knox *et al.*, *Biochem Pharmacol* **37**: 4661–4669, 1988). We show here that 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide can become a species capable of binding to DNA and producing interstrand crosslinks, by a direct, non-enzymatic reaction with either acetyl coenzyme A, butyl and propyl coenzyme A or *S*-acetylthiocholine. Coenzyme A itself cannot produce these effects. The major product of the reaction between the 4-hydroxylamine and thioesters was identified as 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide. However, this compound is not capable of producing the above effects and the major DNA reactive species was a minor product of the reaction. It is proposed that the ultimate, DNA reactive, derivative of CB 1954 is 4-(*N*-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide.

The basis of the selective cytotoxicity of CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) is that this compound can act as a difunctional agent. This is because of its reduction by the enzyme DT diaphorase to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, an agent which can react difunctionally in cells and produce DNA–DNA interstrand crosslinks [1, 2]. This bioactivation was considered to be specific towards the rat Walker 256 carcinoma. However, other rat cell lines are now known to be sensitive towards CB 1954, as predicted by their levels of the activating enzyme, DT diaphorase [3]. However, human cells, even those expressing significant levels of DT diaphorase, are not sensitive towards CB 1954. The reason for their lack of sensitivity is due to differences in the kinetics of CB 1954 reduction between the human and rat forms of DT diaphorase. Although both forms can produce the cytotoxic 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide derivative, the rate of reduction of CB 1954 by the human form of the enzyme is intrinsically much lower [3].

Irrespective of the ability of the cells to bioactivate CB 1954, all the cell types so far examined have a comparable sensitivity towards the reduced 4-hydroxylamino derivative [3]. This compound can produce DNA–DNA interstrand crosslinks in cells but not in naked DNA [1]. It was suggested that in cells there is a further activation step that converts 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to the proximal, DNA crosslinking, cytotoxic species. This notion was supported by the absence of a linear dose-response in crosslinking in cells treated with this compound, consistent with the saturation of this second activation step [1]. An enzymatic esterification and activation of the hydroxylamines formed by metabolism of 4-nitroquinoline-*N*-oxide and *N*-acetylaminofluorene is known to occur [4, 5] and it was proposed that an analogous enzymatic reaction could account for the further activation of the 4-hydroxylamino form of CB 1954 [1].

We now report that 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide can be activated non-enzymatically, to a form capable of reacting with naked DNA to produce interstrand crosslinks, by a direct chemical reaction with acetyl-coenzyme A and other thioesters.

MATERIALS AND METHODS

Materials. All chemicals and reagents were supplied by the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. *S*-Acetylthiocholine iodide was obtained from the Aldrich Chemical Co. (Gillingham, U.K.). CB 1954 and 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide were synthesized or supplied by Dr M. Jarman and Dr L. Davies (ICR).

Preparation of radiolabelled DNA. Cellular DNA

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was radiolabelled by growth of Walker or V79 cells for 24 hr in the presence of [6-^{3}H]thymidine followed by a 2 hr label-free chase period as previously described [6]. DNA was extracted and purified, again as previously described [6].

Determination of DNA interstrand crosslink formation. Labelled DNA obtained as above was washed and dissolved in PBS. The DNA was incubated at 37° for 4 hr in the presence of 50 μM 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide with or without the presence of 1 mM of a thioester. Any resulting DNA interstrand crosslinking was analysed by sedimentation in alkaline sucrose density gradients, all as previously described [7].

The binding of CB 1954 *to naked DNA.* A mixture of [U-^{3}H]CB 1954 (50 μM; 140 μCi/mmol), NADH (500 μM), calf thymus DNA (1 mg/mL), acetyl coenzyme A (1 mM) and the enzyme DT diaphorase (50 μg/mL) was incubated in PBS at 37°. At various times a sample (1 mL) was removed and the DNA precipitated with ethanol. The DNA was then washed extensively (five times) in 70% ethanol, dried and redissolved in 1 mL of water containing DNAase (50 μg/mL) and $MgCl_2$ (100 μM). A sample (750 μL) was removed and its tritium activity determined by scintillation counting whilst another (50 μL) was used to determine the DNA concentration by UV spectroscopy. The reduction of CB 1954 was monitored by HPLC (SCX column [3]).

Preparation of [U-^{3}H]5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. The active form of CB 1954 was radiolabelled by the enzymatic reduction of [U-^{3}H]CB 1954. CB 1954 (100 μM also containing [U-^{3}H]CB 1954 at 1.6×10^4 cpm/nmol), NADH (1 mM) and DT diaphorase (80 μg/mL) were incubated at 37° in PBS. Reduction was monitored by HPLC on an SCX column as previously described [3]. After 2.5 hr the reduction of CB 1954 was essentially complete (>95%) and the enzyme was removed by centrifugal ultrafiltration (Amicon, centricon 10). The radiochemical purity of the reduction product was confirmed as [U-^{3}H]5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (>90%) by reverse phase HPLC [3]. In the presence of NADH the 4-hydroxylamino product was found to be stable and stored as a frozen solution at −20°.

The effect of S*-acetylthiocholine on the binding of* 5-*(aziridin-1-yl)*-4-*hydroxylamino*-2-*nitrobenzamide to DNA.* The time course of binding of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to naked DNA in the presence of various concentrations of *S*-acetylthiocholine was established by use of the radiolabelled 4-hydroxylamino compound as synthesized above. The reaction was started by addition of an aliquot (8 mL) of a solution containing calf thymus DNA (1 mg/mL), NADH (250 μM) and [U-^{3}H]5-(aziridin-1-yl)-4-hydroxyl-amino-2-nitrobenzamide (≈50 μM, 1.6×10^4 cpm/nmol) in PBS to an appropriate volume of *S*-acetylthiocholine (final concn 0–10 mM). The solutions were incubated at 37° and at various times samples were removed and the DNA precipitated with ethanol, washed and its tritium activity determined as above. The reaction mixtures were also analysed at regular intervals by HPLC (SCX column) [3].

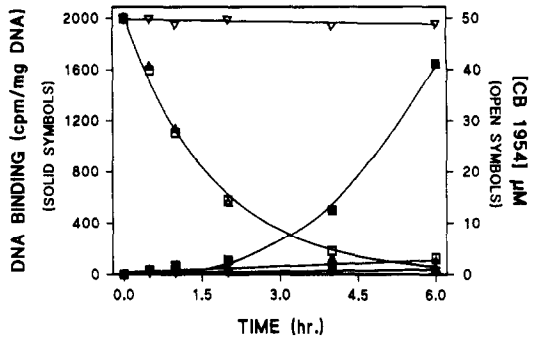

Fig. 1. The reduction of CB 1954 and its binding to naked DNA in the presence of acetyl coenzyme A. No acetyl coenzyme A (△); no enzyme (▽); DT diaphorase (50 μg/mm) and acetyl coenzyme A (1 mM) (□). The solid symbols refer to the resulting DNA binding and the open symbols to CB 1954 reduction. The initial concentration of CB 1954 was 50 μM and NADH (500 μM) was also present as a co-factor for the enzyme. The concentration of DNA was 1 mg/mL and all incubations were done at 37° in PBS.

The products of the reaction between 5-*(aziridin-1-yl)*-4-*hydroxylamino*-2-*nitrobenzamide and* S*-acetylthiocholine.* 5-(Aziridin-1-yl)-4-hydroxyl-amino-2-nitrobenzamide (50 μM, also containing [U-^{3}H]-5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide at 1.6×10^4 cpm/nmol) was reacted with *S*-acetylthiocholine (1 mM) in PBS at 37°. As an internal standard for the HPLC [U-^{3}H]CB 1954 (but not cold CB 1954) to the same activity as the 4-hydroxylamine was also present in the reaction mixture. At various times samples (10 μL) were injected onto a reverse phase HPLC column (Microsorb, ODS-5, 4.6 × 250 mm) and eluted with a methanol gradient (0–40% linear over 30 min, 40–100% linear over 10 min) at 1.0 mL/min. Fractions (0.5 mL) were collected every 30 sec and the tritium activity of each assayed by liquid scintillation counting. Alternatively, a 500 μL sample was injected onto the HPLC and the fractions were collected into tubes containing 0.5 mL of calf thymus DNA (2 mg/mL) and incubated at 37° overnight. Any resulting DNA binding was assayed as above.

RESULTS

Reduction of CB 1954 *and its binding to naked DNA in the presence of acetyl coenzyme A*

CB 1954 was reduced in the presence of naked calf thymus DNA by the enzyme DT diaphorase using NADH as a co-factor. Binding of the drug to DNA was measured by [^{3}H]CB 1954 activity remaining with the DNA after extensive washing. In the absence of the activating enzyme there was no reduction of CB 1954 and little drug binding to DNA (Fig. 1). Addition of the enzyme and the consequent reduction of CB 1954 produced little additional DNA binding. However, the presence of acetyl coenzyme A in the reduction mixture produced a dramatic increase in the amount of drug bound to

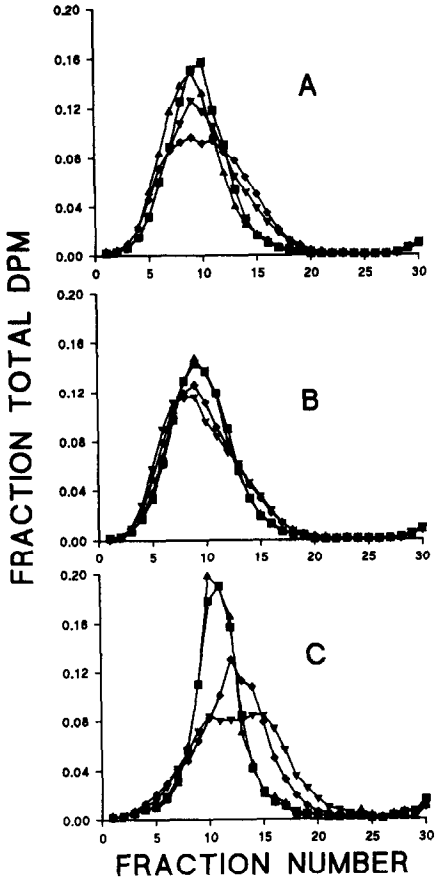

Fig. 2. The formation of DNA interstrand crosslinks in naked DNA by 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and various thioesters. The DNA was treated with: (A) (■) nothing; (▲) 250 μM 4-hydroxylamine; (▼) 50 μM 4-hydroxylamine + 1 mM acetyl coenzyme A; (◆) 250 μM 4-hydroxylamine + 1 mM acetyl coenzyme A; (B) (■) nothing; (▲) 250 μM 4-hydroxylamine + 1 mM coenzyme A; (▼) 250 μM 4-hydroxylamine + 1 mM propyl coenzyme A; (◆) 250 μM 4-hydroxylamine + 1 mM butyl coenzyme A; and (C) (■) nothing; (▲) 1 mM *S*-acetylthiocholine; (▼) 50 μM 4-hydroxylamine + 1 mM *S*-acetylthiocholine; (◆) 250 μM 4-hydroxylamine + 1 mM *S*-acetylthiocholine. All reactions were for 2 hr at 37° in PBS. The direction of sedimentation is from left to right. The crosslink frequencies produced by the various treatments are given in the text.

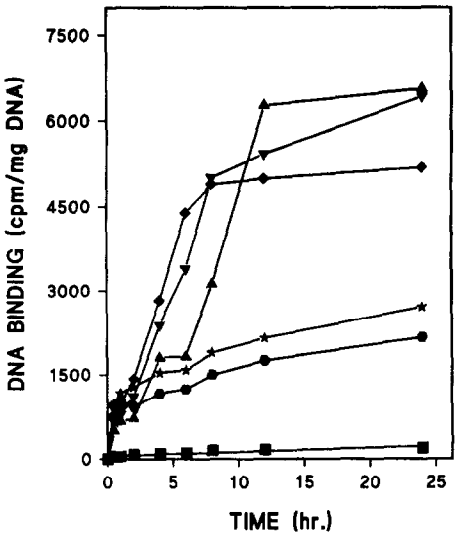

Fig. 3. The binding of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to naked DNA in the presence of *S*-acetylthiocholine. The concentrations of *S*-acetylthiocholine are: 0 (■); 0.5 mM (▲); 1 mM (▼) 2 mM (◆); 5 mM (★); 10 mM (●). The initial concentration of 4-hydroxylamine was 50 μM and of DNA 1 mg/mL. The reactions were carried out at 37° in PBS.

DNA (Fig. 1). Acetyl coenzyme A had no effect on the rate of CB 1954 reduction (Fig. 1).

Thioester activation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and production of DNA interstrand crosslinks

Incubation of either 50 or 250 μM 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide with naked DNA for 2 hr at 37° produced no detectable DNA–DNA interstrand crosslinks in naked calf thymus DNA (Fig. 2A). The addition of 1 mM acetyl coenzyme A in the reaction mixture resulted in an increase in the proportion of the DNA sedimenting further into the alkaline sucrose gradient (Fig. 2A) and this is characteristic of the formation of DNA interstrand crosslinks [7]. Interstrand crosslinks were also formed in the presence of either butyl coenzyme A or propyl coenzyme A (Fig. 2B) and *S*-acetylthiocholine (Fig. 2C) but not by coenzyme A itself (Fig. 2B). The actual crosslink frequencies were determined mathematically from the above sedimentation profiles [7]. Under the standard conditions used (250 μM 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, 1 mM thioester reacted with 1 mg/mL calf thymus DNA for 2 hr at 37°) the crosslink frequency (expressed per 10^9 daltons of DNA) was determined to be 26.6 in the presence of acetyl coenzyme A, 15.4 and 21.5 in the presence of the butyl and propyl derivatives, respectively, and 31.8 when *S*-acetylthiocholine was present. At a concentration of 50 μM 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide the presence of 1 mM acetyl coenzyme A produced 13.3 crosslinks per 10^9 daltons of DNA and 1 mM *S*-acetylthiocholine gave a value of 39.6.

The binding of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to naked DNA in the presence of S-acetylthiocholine

Figure 3 illustrates the time course of binding of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to naked calf thymus DNA in the presence of various concentrations of *S*-acetylthiocholine. In the absence of *S*-acetylthiocholine there was little binding to DNA by 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. In the presence of *S*-acetylthiocholine there was a dramatic increase

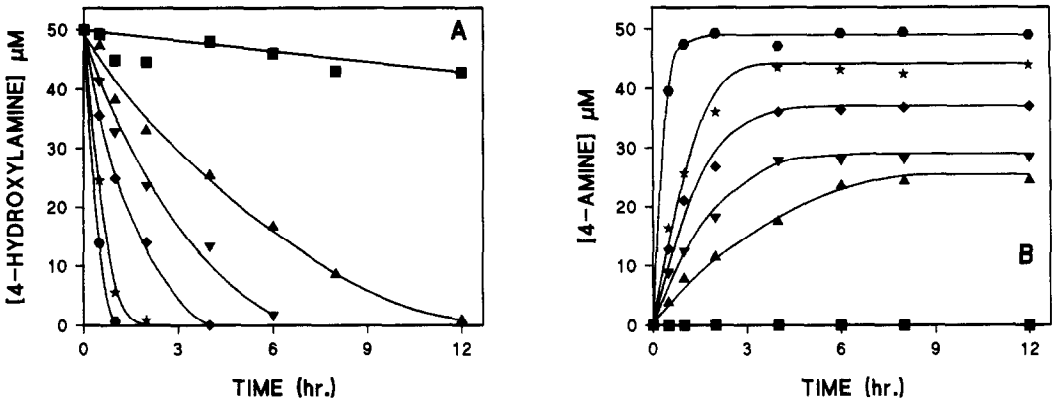

Fig. 4. The loss of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (A) and the gain of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (B) during the reaction between the 4-hydroxylamine and various concentrations of *S*-acetylthiocholine. The conditions and symbols are as in Fig. 3.

in the amount of radiolabel bound to DNA and this increased with time. However, increasing the concentration of *S*-acetylthiocholine above 1 mM resulted in a decrease in the total binding to DNA of the labelled 4-hydroxylamine, although the initial rate of binding was probably faster. The maximum binding achieved with 1 mM *S*-acetylthiocholine represented about 0.8% of the total radioactivity in the reaction mixture. Increasing concentrations of *S*-acetylthiocholine resulted in an increased rate of loss of the 4-hydroxylamine from the reaction mixture (Fig. 4A) and in the rate of formation and total amount of a major product of this reaction which has been identified as 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide (see below) (Fig. 4B). The initial rates of formation of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide were similar to the rates of loss of the 4-hydroxylamine.

The products of the reaction between 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and S-acetylthiocholine

The reaction between *S*-acetylthiocholine and 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide was followed by HPLC. The initial concentration of the 4-hydroxylamine was 50 μM and that of the *S*-acetylthiocholine 1 mM. The reaction was monitored both optically and by ^{3}H-labelled 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide using [^{3}H]CB 1954 as an internal standard. From the optical traces (320 nm) only a single major product was seen (data not shown) and this was confirmed by the appearance of a ^{3}H-labelled product (Fig. 5A–C, retention time = 14.5 min). By comparison of its retention time and by its UV absorbance spectrum the major product was identified as 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide. Although a significant number of counts also eluted on the exclusion limit of the column these counts were not accompanied by any significant optical absorbance. The rate of reaction of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide with *S*-acetylthiocholine seemed to be faster in this experiment than that shown in Fig. 4. However, this experiment was not done in the presence of DNA and the reaction mixture was less viscous.

The ability of any reaction products to bind to DNA was also measured. As shown in Fig. 5D there is a major DNA binding product (retention time = 7.5 min). This DNA binding species does not correspond to a major product peak and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide did not bind to DNA (Fig. 5A–D).

DISCUSSION

CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) is, chemically, a monofunctional alkylating agent. However, an exceptional and selective activity (atypical of a monofunctional compound) of CB 1954 towards the Walker tumour [8] led to extensive studies on the mechanism of its cytotoxicity (see Refs 9 and 10). The basis of the selective action of CB 1954 is now known. It is the result of the aerobic bioreduction of the drug to a difunctionally reacting species by the enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone)) [1, 2]. The enzyme catalyses the reduction of the 4-nitro group of CB 1954 to the corresponding 4-hydroxylamino compound [1]. This compound, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is the active form of CB 1954. It is toxic to cells resistant to CB 1954 and produces interstrand crosslinks in their DNA [1]. However, in contrast to its ability to crosslink DNA in cells, this compound cannot produce these links in isolated naked DNA and a second activation step to convert the 4-hydroxylamine to the proximal crosslinking species was proposed [1].

By analogy with the enzymatic esterification and activation of the hydroxylamines formed by the metabolism of 4-nitroquinoline-*N*-oxide (4-NQO) and *N*-acetylaminofluorene (AAF) [4, 5] it was thought that an analogous enzymatic reaction could account for the further activation of the 4-hydroxylamino form of CB 1954. The activation of 4-hydroxylaminoquinoline-*N*-oxide can be mediated by seryl-tRNA synthetase or other aminoacyl-tRNA

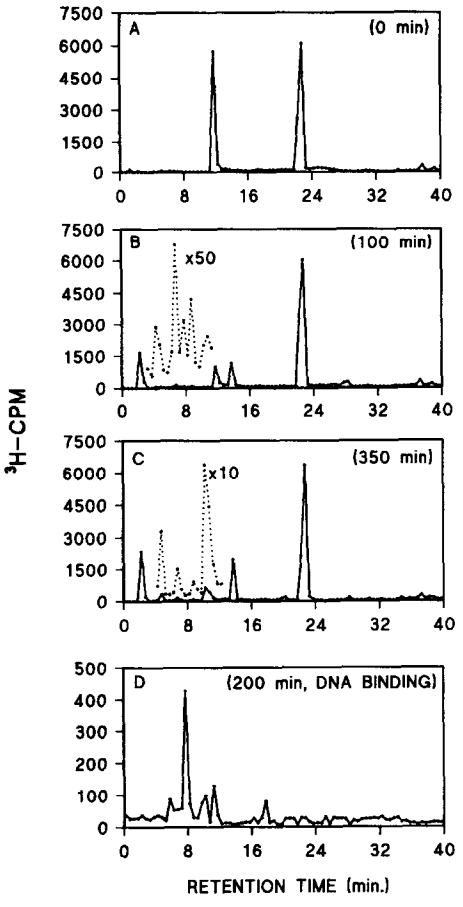

Fig. 5. The products of the reaction between 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (50 μM) and *S*-acetylthiocholine (1 mM) for (A) 0 min; (B) 100 min; (C) 350 min. (D) Illustrates the ability of the products formed after 200 min to bind to DNA. Reaction and separation conditions are as described in the text. The peak times of the other standards were: 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, 11.8 min; 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide, 13.7 min; 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide, 26.2 min and 2-amino-5-(aziridin-1-yl)-4-nitrobenzamide, 24.6 min.

synthetases [11]. We attempted to activate 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (in the presence of amino acids and ATP) by a commercially available mixture of these enzymes without success (unpublished data). Although the above enzymatic conjugation may be important in the ultimate activation of 4-NQO other acetyltransferase enzymes, such as acetyl coenzyme A: arylamine-acetyltransferase and *N*-hydroxyarylamine *O*-acetyltransferase are also known to exist. The activation of *N*-hydroxy-AAF by *N,O*-acyltransferase has been demonstrated [12]. The enzyme arylhydroxamic acid *N,O*-acetyltransferase can catalyse the metabolic activation of arylhydroxamic acids by forming an unstable *N*-acetoxyarylamine. This is achieved by intramolecular transfer of the acetyl group from N to O but another arylhydroxamic acid can also act as a donor [13].

To investigate the possible involvement of such enzymes in the further activation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, we fractionated an extract of V79 cells on a gel filtration column and assayed fractions for enhanced DNA binding in the presence of an acetyl donor–acetyl coenzyme A, radiolabelled hydroxylamine being generated *in situ* by a combination of [^{3}H]CB 1954, DT diaphorase and NADH. No peak of enhanced binding activity was detected, but all fractions, even those collected before the exclusion volume of the column, showed a level of DNA bound radiolabel significantly above background (unpublished data). This result suggested that the presence of an acetyl donor alone could stimulate DNA binding by 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. That this was indeed the case was shown when acetyl coenzyme A and DNA were present during the reduction of CB 1954 by DT diaphorase. Little DNA binding was observed in the absence of either DT diaphorase or acetyl coenzyme A but this was dramatically increased when both were present, indicating a requirement for nitroreduction and an acetyl donor. Acetyl coenzyme A had no effect on the rate of CB 1954 reduction but it was noted that the time course of binding lagged behind the reduction of CB 1954. This could indicate either a slow reaction between the 4-hydroxylamine derivative of CB 1954 and acetyl coenzyme A or a slow reaction between the activated hydroxylamine and DNA. It is conceivable from this result that acetyl coenzyme A could be interfering with the reduction of CB 1954 by DT diaphorase and producing a product capable of directly reacting with DNA. However, greatly enhanced binding was later observed in the presence of chemically synthesized 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, thus ruling out the above possibility.

If the DNA binding observed above was analogous to the intracellular activation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide then the formation of interstrand crosslinks would be expected to be observed in this naked DNA. Incubation of the 4-hydroxylamine and acetyl coenzyme A in the presence of naked DNA did indeed result in production of the predicted crosslinks. Induction of crosslinks was also observed with other thioesters such as both propyl and butyl coenzyme A and *S*-acetylthiocholine, but no effect was seen with coenzyme A itself. Under the standard conditions used, the different thioesters produced different crosslink frequencies. Propyl and butyl coenzyme A were less effective than acetyl coenzyme A at crosslink formation but the most effective agent examined was *S*-acetylthiocholine which produced significantly more crosslinks than acetyl coenzyme A. The differences in crosslink formation is probably a reflection of the relative reactivity of these thioesters towards 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide or the stability/reactivity of the products of this reaction.

Due to its greater efficacy at crosslink induction, the effect of *S*-acetylthiocholine concentration on induction of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide binding to DNA was investigated. Although the 4-hydroxylamine was lost in a

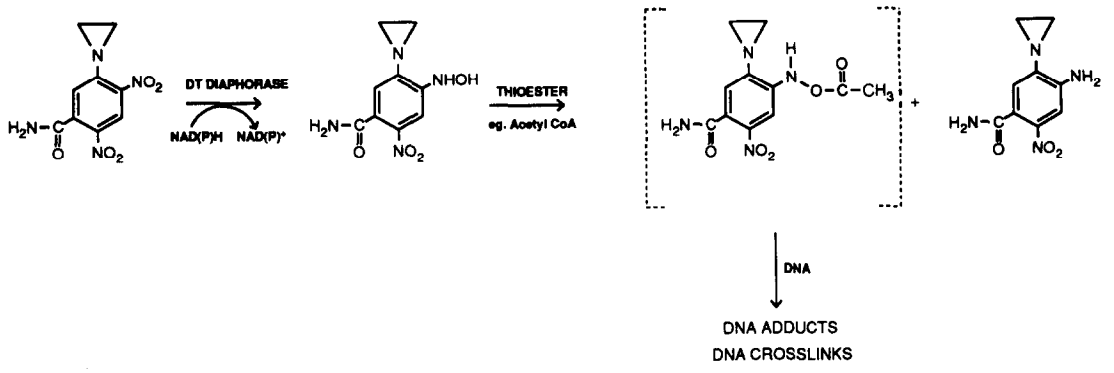

Fig. 6. The bioactivation of CB 1954. The initial step is the reduction of CB 1954 by the enzyme DT diaphorase to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This hydroxylamine derivative can react with thioesters to produce DNA reactive species. It is postulated that this is the *N*-acetoxy derivative. The major product of this reaction is however 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide which does not react readily with DNA. Formation of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide is in competition with the production of DNA binding products (see text for details).

manner dependent upon the concentration of *S*-acetylthiocholine, the total amount of DNA bound 4-hydroxylamine actually decreased when the concentration of the thioester was greater than 1 mM. However, the initial rates of binding did seem to increase with *S*-acetylthiocholine concentration. This suggests that as well as activating 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, *S*-acetylthiocholine can also react with the product of this activation to form a product (perhaps a diacetylated derivative) which does not readily react with DNA. The major product of the reaction between 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and *S*-acetylthiocholine was 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide i.e. the 6-electron reduction product of CB 1954. At high concentrations of *S*-acetylthiocholine most of the hydroxylamine seemed to form this amine and there was much less DNA binding; but under conditions where much more DNA binding was observed the total amount of amine produced was less. This suggests that the formation of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide is a result of the over acetylation of the hydroxylamine discussed above and its formation is in competition with DNA binding. The existence of a competing reaction, the rate of which is dependent on the concentration of an acetylated intermediate, would also explain why fewer crosslinks were observed in naked DNA treated with *S*-acetylthiocholine and 250 μM 4-hydroxylamine than with 50 μM 4-hydroxylamine. It was also noted that DNA binding continued to increase even after the 4-hydroxylamine was effectively exhausted, indicating that the activated hydroxylamine is reasonably stable over a few hours. That the amine derivative can be formed from the hydroxylamine by this reaction is of interest because this is the major urinary metabolite of CB 1954 in the rat [14]. We did tentatively identify 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide as a secondary product of CB 1954 reduction by DT diaphorase [1]; however, further work has failed to show any of this product [3] and 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide can be produced in about 100% yields. It is possible that isolation of some acetyl coenzyme A (or similar chemicals) with DT diaphorase during purification of the enzyme could account for the initial observation.

The actual reaction mechanism by which 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide is formed is unknown. Whatever its mechanism of formation this product is not responsible for the observed binding or crosslinking of naked DNA. The DNA binding ability of the products of the reaction between 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and *S*-acetylthiocholine were assayed after fractionation by HPLC. Under the conditions of this experiment only about half the final reaction products could be accounted for as the 4-amino derivative. Of the remainder most seemed to be eluting on the void volume of the HPLC column without any accompanying optical absorbance at 320 nm or ability to bind to DNA. This could indicate either fragmentation of the molecule or a molecular rearrangement such that some of the tritium was lost to water. There was a major peak of DNA binding activity but this did not correspond to a major product of the reaction. As well as illustrating the efficiency of its reaction with DNA, the fact that it was not a major product would suggest that it is either unstable or it is only formed in low yields. However, as discussed above the continuing binding after depletion of the 4-hydroxylamine would suggest a reasonable stability for this product.

The DNA binding species, and thus the ultimate crosslinking derivative of CB 1954, was not identified. However, Fig. 6 illustrates a proposed scheme which assumes an acetoxy derivative of the hydroxylamine to be formed and that this is the DNA reactive product. Such a scheme would again be directly analogous to the products formed by the enzymatic activation of *N*-hydroxy-AAF and 4-hydroxylaminoquinoline-*N*-oxide, where the relevant acetoxy derivatives are capable of reacting with naked DNA.

For both these acetoxy derivatives the major DNA adduct appears to be with the C8 position of deoxyguanosine [15, 16]. We have evidence that the activated form of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide does also react predominantly with the C8 position of deoxyguanosine (Davies, Jarman and Knox, unpublished data) which might be predicted for an acetoxy derivative. In the case of CB 1954, monofunctionally bound as above, the aziridine group is now available to react, introduce difunctional damage into DNA and produce the observed crosslinks. It is the activation of this molecule from a poor monofunctional alkylating agent to a potent difunctional agent that accounts for the dramatic sensitivity of cell lines capable of reducing CB 1954. These cell lines can be over 100,000-fold more sensitive (on a dose basis) than cells unable or poorly capable of carrying out the bioreduction [3, 17] and CB 1954 can actually cure the Walker tumour in rats [8].

In summary, we have demonstrated that 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide can be made into a DNA reactive and crosslinking species by a direct, non-enzymatic reaction with acetyl coenzyme A or other thioesters. The fact that this final activation step in the bioactivation pathway occurs non-enzymatically with a common and vital cellular component would explain the similar sensitivities of a number of cell lines towards 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide [3] and suggests that resistance to CB 1954 cannot occur by amelioration of this step. Thus, it remains that the reduction of CB 1954 by DT diaphorase defines cellular sensitivity to this agent.

Although the use of CB 1954 in the therapy of human tumours will be limited by the fact that the human form of DT diaphorase intrinsically reduces this drug at a slow rate [3], the fact that all the steps in its activation are now understood make it more likely that a CB 1954 analogue can be synthesized which could regenerate in specific human tumours the dramatic antitumour activity of CB 1954 observed in the rat Walker tumour.

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REFERENCES

1. 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.
2. Knox RJ, Boland M, 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-nitrobenzamideisaformofNAD(P)Hdehydrogenase (quinone) EC 1.6.99.2). *Biochem Pharmacol* **37**: 4671–4677, 1988.
3. Boland MP, Knox RJ and Roberts JJ, 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). *Biochem Pharmacol* **41**: 867–875, 1991.
4. Tada M and Tada M, Seryl-t-RNA synthetase and activation of the carcinogen 4-nitroquinoline 1-oxide. *Nature* **255**: 510–512, 1975.
5. DeBraum JR, Miller EC and Miller JA, *N*-Hydroxy-2-acetyllaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein-(methion-*S*-yl) binding in rat liver. *Cancer Res* **30**: 577–595, 1970.
6. Knox RJ, Friedlos F, Lydall D and Roberts JJ, Mechanism of cytotoxicity of anticancer platinum drugs; evidence that *cis*-diamminedichloroplatinum(II) and *cis*-diamine-1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res* **46**: 1972–1979, 1986.
7. Roberts JJ and Friedlos F, Quantitative estimation of cisplatin-induced DNA interstrand cross-links and their repair in mammalian cells: relationship to toxicity. *Pharmac Ther* **34**: 215–246, 1987.
8. 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. *Chem Pharmacol* **18**: 1519–1527, 1969.
9. Workman P, White RAS and Talbot K, CB 1954 revisited I. Disposition kinetics and metabolism. *Cancer Chemother Pharmacol* **16**: 1–8, 1986.
10. 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.
11. Tada M and Tada M, Metabolic activation of 4-nitroquinoline 1-oxide and its binding to nucleic acid. In: *Fundamentals in Cancer Prevention*. (Eds. Magee PN, Takayama S, Sugimura T and Matsushima T), pp. 217–228. University Park Press, Baltimore, U.S.A., 1976.
12. Thorgeirsson SS, Wirth PJ, Staiano N and Smith CL, Metabolic activation of 2-acetylaminofluorene. *Adv Exp Med Biol* **136B**: 897–919, 1982.
13. King CM, Mechanisms of reaction, tissue distribution, and inhibition of arylhydroxamic acid acyltransferase. *Cancer Res* **34**: 42–55, 1974.
14. Jarman M, Melzack DH and Ross WCJ, The metabolism of the anti-tumour agent 5-(1-aziridinyl)-2,4-dinitrobenzamide (CB 1954). *Biochem Pharmacol* **25**: 2475–2478, 1976.
15. Bailleul B, Galiegue S and Loucheux-Lefebvre MH, Adducts from the reaction of *O*,*O*′-diacetyl or *O*-acetyl derivatives of the carcinogen 4-hydroxyaminoquinoline 1-oxide with purine nucleosides. *Cancer Res* **41**: 4559–4565, 1981.
16. Howard PC, Casciano DA, Beland FA and Shaddock JG, The binding of *N*-hydroxy-2-acetylaminofluorene to DNA and repair of the adducts in primary rat hepatocyte cultures. *Carcinogenesis* **2**: 97–102, 1981.
17. 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.