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CAFFEINE, AMINOIMIDAZOLECARBOXAMIDE AND DICOUMAROL, INHIBITORS OF NAD(P)H DEHYDROGENASE (QUINONE) (DT DIAPHORASE), PREVENT BOTH THE CYTOTOXICITY AND DNA INTERSTRAND CROSSLINKING PRODUCED BY 5-(AZIRIDIN-1-YL)-2,4-DINITROBENZAMIDE (CB 1954) IN WALKER CELLS

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Abstract—A form of NAD(P)H dehydrogenase (quinone) (DT diaphorase, menadione reductase (NMOR), phylloquinone reductase, quinone reductase, EC 1.6.99.2) has been isolated from Walker 256 rat carcinoma cells. This enzyme can convert 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to a cytotoxic DNA interstrand crosslinking agent by reduction of its 4-nitro group to the corresponding hydroxylamino species (Knox *et al. Biochem Pharmacol*, **37**: 4661–4669 and 4671–4677, 1988). 2-Phenyl-5(4)-aminoimidazole-4(5)-carboxamide and AICA [5(4)-aminoimidazole-4(5)-carboxamide] have previously been reported to be antagonists of the anti-tumour effects of CB 1954. We have shown that both these compounds are inhibitors of the above enzyme and that AICA protects against both the cytotoxicity and the formation of DNA interstrand crosslinks, produced by CB 1954 in Walker cells. Similarly, known inhibitors of NAD(P)H dehydrogenase (quinone) such as dicoumarol, also reduced the cytotoxicity and DNA-interstrand crosslinking of CB 1954 in Walker cells. Caffeine was shown to be a novel inhibitor of NAD(P)H dehydrogenase (quinone) and also elicited the above protective effects. All of the above inhibitors were also shown to potentiate the toxic effects of menadione against the Walker cell. This quinone is known to be detoxified by NAD(P)H dehydrogenase (quinone) and thus emphasises the ability of these compounds to inhibit this enzyme within the cell.

CB 1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] is a monofunctional alkylating agent, which exhibits a highly specific toxicity towards the Walker 256 cell growing either as a tumour in the rat [1] or as a cultured cell [2, 3]. The basis of this selective action of CB 1954 is the formation of DNA interstrand crosslinks in these toxicically-affected cells, but not in non-sensitive cells [3, 4]. Crosslink formation is a result of the bio-activation of the drug, by the specific reduction of the 4-nitro group, to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a difunctionally-reacting species which is toxic to those cells which are resistant to CB 1954 [4]. The enzyme which performs this aerobic reduction of CB 1954 has been isolated, purified and characterised [4, 5]. It has been identified as a form of NAD(P)H dehydrogenase (quinone) (DT diaphorase, EC 1.6.99.2) by comparison of partial protein sequences, coenzymes, substrate and inhibitor specificities and spectroscopic data. This enzyme, commonly called DT diaphorase, catalyses the two-electron reduction of quinones directly to hydroquinones, and is strongly inhibited by agents such as dicoumarol and warfarin. Because hydroquinones can be conjugated to glucuronic acid and excreted from the cell, this enzyme is thought to be primarily involved in quinone detoxification (for a review see Ref. 6). Quinones are cytotoxic because they can also be reduced [by a one-electron reduction, catalysed by enzymes other than NAD(P)H dehydrogenase (quinone)] to the semiquinone form. This free radical can react with oxygen to regenerate the quinone and produce superoxide and other reactive oxygen species. Hence inhibition of NAD(P)H dehydrogenase (quinone), by agents such as dicoumarol, potentiates the cytotoxicity of quinones such as menadione [7].

Two compounds, 2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide and AICA [5(4)-aminoimidazole-4(5)-carboxamide], previously reported to be antagonists of the anti-tumour effects of CB 1954 *in vivo* [8–10] have been shown to be inhibitors of this enzyme [5].

Caffeine is known to be able to potentiate the cytotoxic and chromosome-damaging effects of chemical agents (for a review see Ref. 11). However, in contrast to this potentiating effect, caffeine reduced the cytotoxic effect of 4-nitroquinoline-1-oxide but not of its activated form, 4-hydroxylaminoquinoline-1-oxide [12]. As dicoumarol could

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also produce such protective effects, it was suggested that NAD(P)H dehydrogenase (quinone) was involved in the reductive activation of 4-nitroquinoline-1-oxide [12, 13] and that caffeine could also inhibit this enzyme [12].

We now show that inhibitors of NAD(P)H dehydrogenase (quinone), in contrast to their potentiating effects on quinone toxicity, prevent both the cytotoxicity and DNA interstrand crosslinking ability of CB 1954 in Walker cells. We also report that caffeine is an inhibitor of this enzyme. These results emphasise the role of NAD(P)H dehydrogenase (quinone) in the activation of CB 1954 in this cell line.

MATERIALS AND METHODS

Materials. All chemicals and reagents were supplied by Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. CB 1954 was kindly supplied by Dr D. Wilman (Chemistry-Drug Metabolism Team, ICR).

Purification of NAD(P)H dehydrogenase (quinone) from Walker 256 tumour cells. The purification of this enzyme, from Walker cells, by successive gel filtration and anion exchange HPLC was as previously described [4].

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 [14]. Cells were radiolabelled in their DNA as previously described [3].

Determination of effects of agents on cell survival. The effects of CB 1954 or menadione, in the absence or presence of various inhibitors of NAD(P)H dehydrogenase (quinone), on the survival of Walker cells were determined as previously described [14]. Enzyme inhibitors were added to the cell cultures 10 min prior to either menadione or CB 1954 and removed with the drug, after a 2 hr co-incubation, prior to plating the cells out for their colony forming ability.

Determination of DNA interstrand crosslinking. Walker cells, labelled in their DNA with [^{14}C]thymidine, were treated with 20 μM CB 1954 in the presence or absence of inhibitors of NAD(P)H dehydrogenase (quinone) for 2 hr at 37°. The cells were harvested by centrifugation, washed, resuspended in fresh medium and then left, at 37°, for a further 3 hr. The cells were analysed for DNA crosslinks by sedimentation in alkaline sucrose density gradients, all as previously described [15, 16].

Kinetic and inhibition studies. NAD(P)H dehydrogenase (quinone) activities were assayed by a spectrophotometric method using menadione as a substrate and cytochrome *C* as a terminal electron acceptor as previously described [5]. Values for K_i were derived from Dixon plots of $1/v$ against inhibitor concentration at various concentrations of NADH or menadione and K_i' values from plots of [NADH]/v or [menadione]/v against inhibitor concentration at various concentrations of NADH or menadione, where v is the initial reaction rate. The point of intersection on these plots gives the appropriate constant and the pattern of the plots the type of inhibition, as described by Cornish-Bowden [17].

RESULTS

Inhibitors of NAD(P)H dehydrogenase (quinone)

We have previously shown that inhibition of NAD(P)H dehydrogenase (quinone) by both 2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide and AICA [5(4)-aminoimidazole-4(5)-carboxamide] was complex in that a plot of the reciprocal of the rate of reaction against the inhibitor concentration was non-linear [5]. We have also reported a K_i value of 10 nM for dicoumarol with respect to NADH [5]. Table 1 shows the full kinetic data for the three inhibitors used in this study and also the inhibitor concentrations required to reduce the reaction rate to 10 or 50% of the control rate. Caffeine was found to be a competitive inhibitor of NAD(P)H dehydrogenase (quinone) with respect to NADH (K_i = 1.08 mM) (Fig. 1A and C) and mixed with respect to menadione (K_i = 0.66 mM and K_i' = 3.15 mM) (Fig. 1B and D).

Protection against CB 1954-induced cytotoxicity by inhibitors of NAD(P)H dehydrogenase (quinone)

Dicoumarol, caffeine and AICA all protect against the cytotoxicity of CB 1954 in Walker 256 cells (Fig. 2). Thus co-treatment of Walker cells with 100 nM CB 1954 (which alone reduced cell survival to about 0.15%) and any of these compounds produced a dose-dependent reduction in cell death when present at the same time as CB 1954. No such protective effects were observed when the inhibitors were added to the cells for 2 hr, then removed immediately prior to CB 1954 treatment or when the inhibitor was added to the cells for 2 hr after removal of the CB 1954 (data not shown). Dicoumarol, the most potent inhibitor of NAD(P)H dehydrogenase (quinone) was, on a dose basis, also the most effective protective agent, followed by caffeine, with AICA being the least effective. This ranking was in order of their ability to inhibit NAD(P)H dehydrogenase (quinone).

Potentiation of menadione-induced cytotoxicity by inhibitors of NAD(P)H dehydrogenase (quinone)

All of the above inhibitors potentiated the cytotoxicity of menadione in a dose dependent manner (Fig. 3). The potentiating effect of the three compounds was, as for their protective effects against CB 1954 cytotoxicity, in the order of their ability to inhibit NAD(P)H dehydrogenase (quinone).

Prevention of CB 1954-induced DNA interstrand crosslinking by NAD(P)H dehydrogenase (quinone) inhibitors

Incubation of Walker cells in the presence of CB 1954 results in the generation of interstrand crosslinks in the DNA of the Walker cells. Thus the presence of 20 μM CB 1954, for 2 hr, resulted in an increase in the proportion of the Walker cell DNA sedimenting further into the alkaline sucrose gradient (Fig. 4A), characteristic of DNA interstrand crosslink formation [15, 16]. Removal of the drug and incubation for a further 3 hr, resulted in a still

Table 1. The ability of various compounds to inhibit the reduction of menadione by NAD(P)H dehydrogenase (quinone)

Inhibitor	Varying	K_i	K_i'	Type	Concentration	
					To reduce the reaction rate to 50%	10%
Dicoumarol	NADH	10 nM	20 nM	Mixed		
	Menadione	10 nM		Noncompetitive	15 nM	160 nM
Caffeine	NADH	1.08 mM	—	Competitive		
	Menadione	0.66 mM	3.15 mM	Mixed	1.35 mM	11.2 mM
AICA	NADH or Menadione	—	—	Complex	1.39 mM	1.91 mM

All measurements were carried out at 37° as previously described [5]. Standard concentrations of menadione and NADH were 10 μM and 500 μM respectively.

further increase in the proportion of the DNA which was crosslinked (Fig. 4A). This is presumably because the initial reaction of the crosslinking species with DNA is monofunctional which is then followed by a time-dependent reaction of the second arm producing the observed crosslinks. Such a reaction mechanism is known to occur with carboplatin [18] and the effect is also observed with other difunctional compounds, such as cisplatin and chloroethylnitrosoureas, both *in vivo* and *in vitro* [18, 19]. This enhanced crosslinking allowed a much greater contrast between the untreated, CB 1954 treated and inhibitor and CB 1954 co-treated samples to be made. Co-exposure of the cells to CB 1954 and either 500 μM dicoumarol (Fig. 4B), 2 mM caffeine (Fig. 4C) or 2 mM AICA (Fig. 4D) resulted in a substantial decrease in the proportion of the crosslinked DNA. When Walker cells were treated with any of these inhibitors in the absence of CB 1954, there was no effect on the resulting sedimentation profile of the DNA, which could not be distinguished from that of untreated control cells (data not shown).

DISCUSSION

These results confirm our previous conclusions that the sensitivity of Walker cells towards the monofunctional alkylating agent, CB 1954, is a consequence of their ability to metabolise this compound to a difunctional agent which can induce DNA interstrand crosslinks and that the enzyme that catalyses this reduction is a form of NAD(P)H dehydrogenase (quinone). Activation of CB 1954 occurs by reduction of its 4-nitro group to the corresponding hydroxylamine derivative, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a cytotoxic compound capable of forming DNA interstrand crosslinks in cells [3–5]. The formation of this compound in Walker cells and the consequent induction of DNA crosslinks accounts for the highly specific cytotoxic action of CB 1954 towards these cells. The fact that CB 1954 is activated enzymatically also accounts for the observation, from cell hybridization studies, that unlike the sensitivity of Walker cells to difunctional alkylating agents, the sensitivity to CB 1954 was dominant when a Walker cell was fused with a CB 1954 resistant cell [2].

The identification of NAD(P)H dehydrogenase (quinone) as the enzyme responsible for the bioactivation of CB 1954 in Walker cells was based initially on the comparison of partial protein sequences with the sequence (deduced from the cloned mRNA) of the enzyme as isolated from rat liver [5]. The identification was further confirmed by the fact that the enzyme from Walker cells was biochemically indistinguishable from NAD(P)H dehydrogenase (quinone). Thus, the Walker cell enzyme could reduce menadione and CB 1954 in air using either NADH or NADPH as a co-enzyme, and both these reactions were inhibited by known inhibitors of NAD(P)H dehydrogenase (quinone) such as dicoumarol and warfarin [5]. Interestingly, two compounds, AICA and phenyl-AICA, known to diminish the anti-tumour effects of CB 1954 *in vivo* [8–10], by a mechanism not involving decreased drug uptake into Walker cells [9], were also shown to be inhibitors of the Walker cell enzyme [5]. The anticoagulant dicoumarol was shown to be a mixed inhibitor with respect to NADH and noncompetitive with respect to menadione, the K_i (as previously reported) being 10 nM. Dicoumarol has routinely been used as a diagnostic inhibitor of NAD(P)H dehydrogenase (quinone). However it is now known not to be completely specific for this enzyme in that it can also uncouple oxidative phosphorylation [20] and inhibit a form of UDP-glucuronosyltransferase [21]. It has usually been reported to be a competitive inhibitor (with respect to NAD(P)H) of NAD(P)H dehydrogenase (quinone) with K_i values in the range 50 pM to 200 nM (for a review see Ref. 6); however mixed inhibition has also been observed [22, 23].

The purine precursor AICA inhibits the Walker cell enzyme and this inhibition could explain the antagonistic effect of this compound towards the anti-tumour action of CB 1954 *in vivo*. No kinetic constants could be derived for this compound since the plot of the rate of reaction against AICA concentration was nonlinear [5]. Complex inhibition is not unexpected for NAD(P)H dehydrogenase (quinone) because this enzyme binds two substrates (the electron donor and the electron acceptor) and there is thought to be a co-operative interaction between these two binding sites [6].

Caffeine is shown, for the first time, to be an inhibitor of NAD(P)H dehydrogenase (quinone);

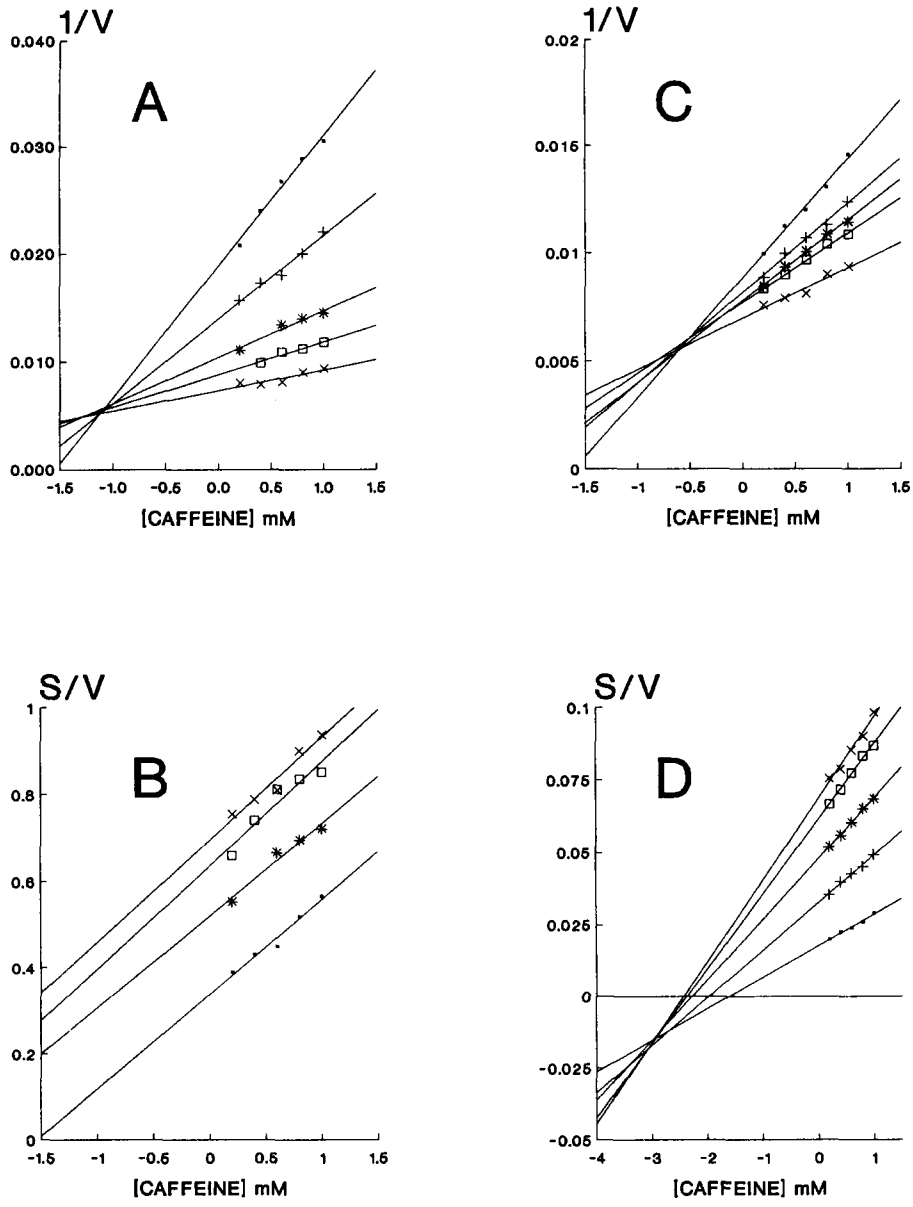

Fig. 1. Inhibition by caffeine of menadione reduction by NAD(P)H dehydrogenase (quinone). Determination of (A) K_i from plots of $1/v$ against caffeine concentration at various concentrations of NADH and (B) K_i' from plots of $[NADH]/v$ against caffeine concentration at various concentrations of NADH, where v is the initial reaction rate, (•10 μM; +25 μM; *50 μM; □75 μM; ×100 μM NADH). The concentration of menadione was constant at 10 μM. Determination of (C) K_i and (D) K_i' at various concentrations of menadione, (•2 μM; +4 μM; *6 μM; □8 μM; ×10 μM menadione). The concentration of NADH was constant at 500 μM. All determinations were made at 37°, with cytochrome c as terminal electron acceptor, as previously described [5]. Initial reaction rates (v) were measured as μmoles cytochrome c reduced per minute. The inhibition was competitive with respect to NADH and mixed with respect to menadione, as determined from the pattern of the plots [17].

the inhibition was competitive with respect to NADH and mixed with respect to menadione. That caffeine might be an inhibitor was suggested by a report [12] that caffeine protected against formation of the mutagen 4-hydroxylaminoquinoline-1-oxide from 4-nitroquinoline-1-oxide and NAD(P)H dehydrogenase (quinone) was implicated in this reductive activation [12, 13]. An equivalent effect, *in vivo*, could account for reports that the transformation of cells by 4-nitroquinoline-1-oxide and the carcinogenic effects of this compound, could be inhibited by caffeine [24–26].

It would be predicted from these results that inhibition of NAD(P)H dehydrogenase (quinone), by the above compounds, would protect Walker cells from the cytotoxic effects of CB 1954 by inhibiting

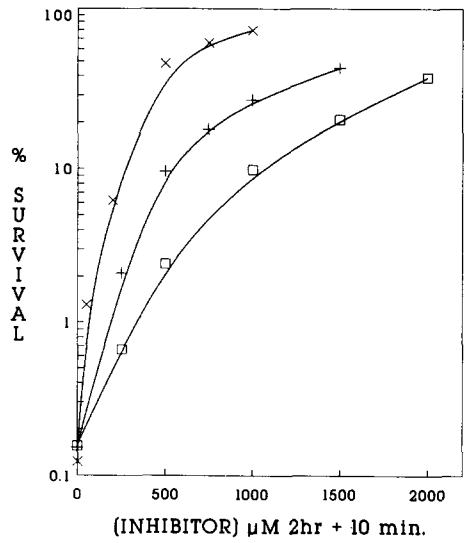

Fig. 2. The effects of inhibitors of NAD(P)H dehydrogenase (quinone) on the survival of Walker cells exposed to CB 1954. Cells were treated for 2 hr, at 37°, with 100 nM CB 1954 in the presence of various concentrations of either (×) dicoumarol, (+) caffeine or (□) AICA and then assayed for colony forming ability. All inhibitors were added to the cells 10 min prior to the CB 1954.

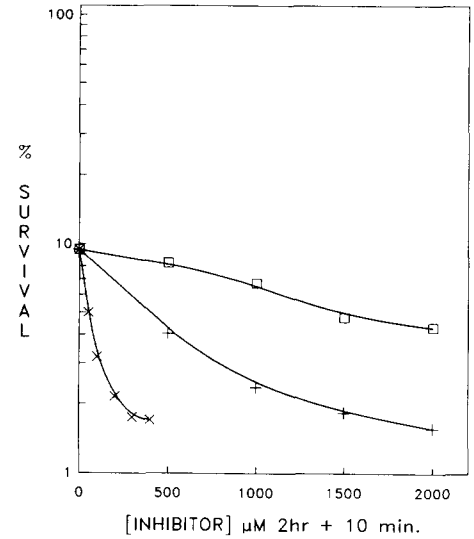

Fig. 3. The effects of inhibitors of NAD(P)H hydrogenase (quinone) on the survival of Walker cells exposed to menadione. Cells were treated for 2 hr, at 37°, with 20 μM menadione in the presence of various concentrations of either (×) dicoumarol, (+) caffeine or (□) AICA and then assayed for colony forming ability. All inhibitors were added to the cells 10 min prior to the CB 1954.

the formation of the active species 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. All three inhibitors produced a dose-dependent reduction in cell death, but only when they were present at the same time as CB 1954, consistent with this protective action being due to the inhibition of the activating enzyme as opposed to other mechanisms. Emphasising that these compounds were indeed acting by inhibiting NAD(P)H dehydrogenase (quinone) in the cell, the same three compounds potentiated the cytotoxicity of menadione, a quinone compound that will be detoxified by this enzyme.

Both the protective and potentiating action of these inhibitors ranked in order of their relative ability to inhibit NAD(P)H dehydrogenase (quinone). However dicoumarol appeared to be relatively less effective as a protective or potentiating agent than might be predicted from its ability to inhibit the enzyme. Thus under the assay conditions using the isolated, pure, enzyme almost 100,000-fold less dicoumarol is required to inhibit the enzyme to 50% of the control rate than either caffeine or AICA, whilst it is apparent that dicoumarol is only about 10-fold more effective, with respect to the protection against CB 1954 cytotoxicity or potentiation of menadione toxicity, than the other two inhibitors. This apparent discrepancy may be because, as we have shown, these three compounds differ in their mechanism of inhibition or because their uptake into cells may be different. Furthermore, dicoumarol is known to bind strongly to albumin [20, 23] and therefore probably to other proteins, which would reduce its effective concentration.

The activation of CB 1954 by Walker cells results in the generation of a difunctional compound, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. In contrast to CB 1954, a monofunctional alkylating agent, this compound possesses two centres which can react with DNA and, diagnostically, form DNA interstrand crosslinks, even in cell lines insensitive to the parent compound. As we have previously discussed [3–4], this activation to a difunctional species fully accounts for the remarkable sensitivity of Walker cells to CB 1954. Hence inhibition of NAD(P)H dehydrogenase (quinone) in these cells should not only result in a decreased cytotoxic effect of CB 1954 but also reduce the amount of DNA interstrand crosslinking produced. That this was indeed the case was shown by alkaline sucrose gradient sedimentation analysis. All three inhibitors markedly reduced the amount of crosslinked DNA in the CB 1954 co-treated cells, relative to that in those cells treated with CB 1954 alone. This decrease in crosslinking would account for the observed increase in cell survival.

In summary, our findings support the notion that, in Walker cells, CB 1954 is activated to a difunctional form by its reduction by the enzyme NAD(P)H dehydrogenase (quinone). Thus, known inhibitors of NAD(P)H dehydrogenase (quinone) have been shown to inhibit the CB 1954 activating enzyme isolated from Walker cells and compounds known to protect against the anti-tumour action of CB 1954, also inhibit this enzyme. Caffeine was also shown to be an inhibitor of NAD(P)H dehydrogenase (quinone) as isolated from Walker cells. All of the above inhibitors not only protected against the cytotoxicity of CB 1954 in Walker cells, presumably by inhibiting the reduction of the drug to a difunctional, DNA crosslinking species, but also potentiated the toxicity of menadione against which NAD(P)H dehydrogenase (quinone) would normally protect.

An enzyme, isolated from the Walker rat tumour, that would normally be considered to be involved in

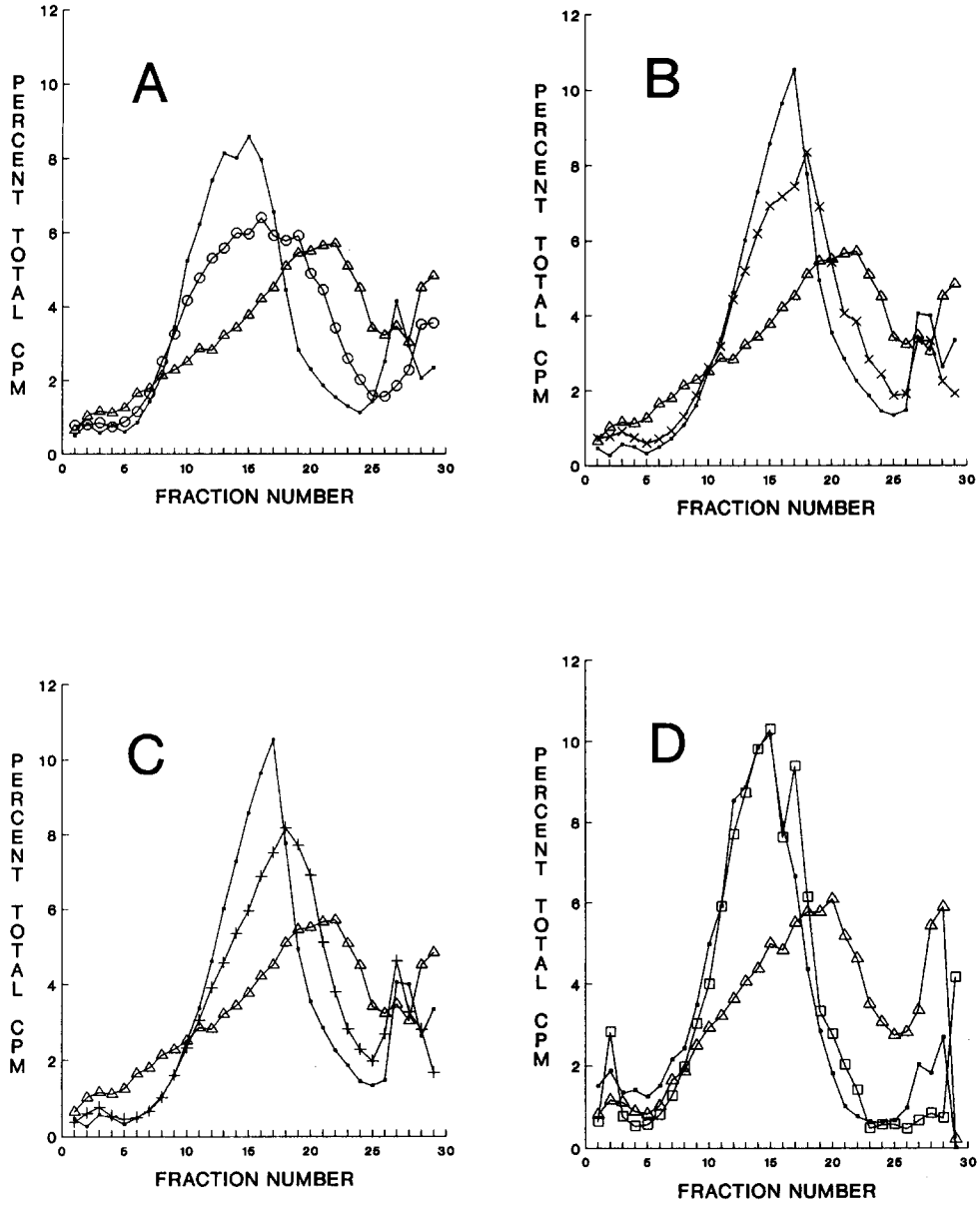

Fig. 4. The effects of inhibitors of NAD(P)H dehydrogenase (quinone) on the formation of DNA interstrand crosslinks by CB 1954 in Walker cells. Cells were treated with 20 μM CB 1954 for 2 hr (A), at 37°, in the presence of either (B) 500 μM dicoumarol, (C) 2 mM caffeine or (D) 2 mM AICA and crosslink formation analysed, after a further 3 hr incubation in the absence of any agent, by sucrose gradient sedimentation. DNA from (●) control, untreated cells; (○) after 20 μM CB 1954 for 2 hr; (△) the same cells 3 hr later; co-treated with (×) 500 μM dicoumarol, (+) 2 mM caffeine or (□) 2 mM AICA. The direction of sedimentation is from left to right.

drug detoxification has been demonstrated to be also capable of bioactivating another compound to a cytotoxic species. It is therefore important to ascertain if this property of the Walker enzyme is common to other forms of NAD(P)H dehydrogenase (quinone) particularly the human enzyme which is known to be homologous to that of the rat (and Walker cell) protein [27]. Now that the basis of the selective action of CB 1954 is understood, it is possible that such bioactivatable compounds may have a place in the chemotherapy of specific human tumours.

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