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ACTIVATION AND DEACTIVATION OF QUINONES CATALYZED BY DT-DIAPHORASE. EVIDENCE FOR BIOREDUCTIVE ACTIVATION OF DIAZIQUONE (AZQ) IN HUMAN TUMOR CELLS AND DETOXIFICATION OF BENZENE METABOLITES IN BONE MARROW STROMA

D. ROSS and D. SIEGEL

Molecular Toxicology and Environmental Health Sciences Program, School of Pharmacy, University of Colorado, Boulder, CO 80309, USA

N.W. GIBSON, D. PACHECO

Lab. Pharmacology, AMC Cancer Research Center, Denver CO 80214, USA

D.J. THOMAS, M. REASOR and D. WIERDA

Dept. Pharmacology and Toxicology, West Virginia University Health Sciences Center, Morgantown, WV 26506, USA

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Bioactivation of diaziquone (AZQ) in HT-29 human colon carcinoma cells and detoxification of benzene metabolites in bone marrow stromal cells were used as examples of the potential role of DT-diaphorase in both activation and deactivation processes. HT-29 cell cytosol contained high levels of DT-diaphorase activity and removed AZQ in the presence of either NADH or NADPH. Prior boiling of cytosol, omission of NADH or NADPH or inclusion of dicoumarol, an inhibitor of DT-diaphorase, inhibited removal of AZQ. AZQ-inouced cytotoxicity in HT-29 cells was also inhibited by dicoumarol. Chemical reduction of AZQ in a cell free system enhanced formation of a GSH conjugate of AZQ. Two of the major cell types in bone marrow stroma are macrophages and fibroblastoid stromal cells. A fibroblastoid cell line derived from stromal cells contained approximately fourfold higher levels of DT-diaphorase than macrophages. Inclusion of dicoumarol in incubations containing ^{14}C-hydroquinone and the respective stromal cell type, significantly increased covalent binding of radiolabel to macromolecules in stromal fibroblasts but not in macrophages.

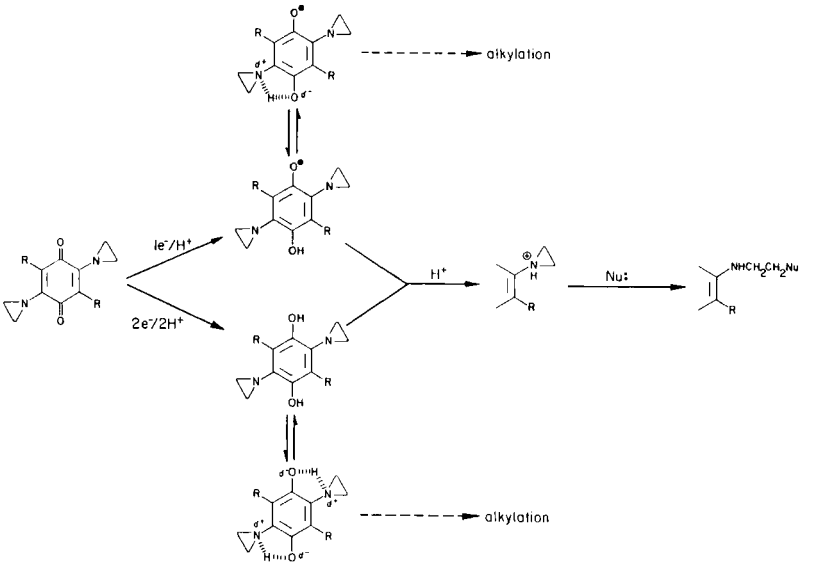
KEY WORDS: DT-Diaphorase, Quinone reductase, Diaziquone (AZQ), Benzene metabolism, Hydroquinone, Bone marrow stroma.

INTRODUCTION

DT-diaphorase (NADPH-quinone-oxidoreductase) is an obligate two-electron reductase which reduces quinones to hydroquinones.[1] This process is usually considered as a detoxification step since two-electron reduction bypasses semiquinone generation, thus preventing a direct one-electron reduction and the subsequent generation of active oxygen species.[2,3] In addition, quinones are electrophilic species and, dependent

on structure, may alkylate nucleophiles while hydroquinones are more hydrophilic and can conjugate with sulfate or glucuronide and are thus more readily excreted. Reactions of hydroquinone derivatives will, however, depend on the chemical structure of the compound and the enzymology of the system under study. Hydroquinones may autoxidize generating reactive oxygen species[4] and in the case of certain antitumor quinones such as AZQ and substituted naphthoquinones, reduction to their hydroquinone derivatives may facilitate the generation of alkylating species.[5,6] Thus, DT-diaphorase may, in certain cases, contribute to bioactivation of quinones, and aspects of the role of DT-diaphorase in activation and deactivation processes have been discussed.[7]

Chemical reduction of the antitumor quinone AZQ favors protonation, which leads to aziridine ring opening and subsequent alkylation reactions (Scheme 1).[6,8-11]. Either



SCHEME I Reductive activation of AZQ[6,8-11,14]

one- or two-electron reduction or acidic conditions facilitate aziridine ring opening and AZQ-induced alkylation reactions. Increased DNA damage has been observed after reduction of AZQ in cell-free systems[12-14] and in tumor cell systems the cytotoxicity of AZQ was found to correlate with the extent of DNA interstrand crosslinking.[15] The role of DT-diaphorase in bioreductive activation of AZQ however, remains unclear.

The mechanisms underlying benzene-induced myelotoxicity are complex but may also involve quinonoid metabolites.[16,17] The major hepatic metabolites of benzene are phenol, catechol and hydroquinone which can undergo peroxidatic oxidation in bone marrow to generate reactive quinones.[18-21] DT-diaphorase in bone marrow may therefore contribute to detoxification of benzene metabolites.

In this manuscript we present preliminary data which suggests that DT-diaphorase is involved in bioactivation of AZQ in human tumor cells and detoxification of benzene metabolites in bone marrow stroma.

MATERIALS AND METHODS

Materials

AZQ was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institute of Health, Bethesda, Maryland 20014. ^{3}H-Glutathione (glycine-2-^{3}H), 1149 mCi/mmol) was purchased from NEN Research Products. Wilmington DE. ^{14}C-Hydroquinone (UL, 22.2 mCi/mmol) was obtained from Wizard Laboratories, Davis CA 95616.

Human colon carcinoma cells HT-29 cells have been passaged in this laboratory for a number of years and clonogenic assays were performed as described previously.[22]

Bone marrow macrophages and a fibroblastoid stromal cell line were obtained from male B6C3F1 mice and cultured as previously described.[23,24]

DT-diaphorase assays were performed by measuring dicoumarol-inhibitable reduction of dichlorophenolindophenol as described previously.[1,25]

Covalent binding Macrophages and fibroblastoid stromal cells were exposed to 0.01 mM hydroquinone $\pm$ 0.02 mM dicoumarol in serum-free medium for 3 h at 37°C. Cells were washed and removed from tissue culture dishes by scraping, collected in 3 ml of phosphate-buffered saline and transferred to tubes where the reaction was stopped by addition of perchloric acid (6% v/v final concentration). Acid-insoluble macromolecules were then washed according to Jollow *et al.*,[26] solubilized, and radioactivity bound covalently determined by scintillation counting.

HPLC analyses were performed using a Supelco C18 5 μm reverse phase column. Gradient elutions were performed using ammonium acetate 50 mM, pH = 6.2 (solution A) and methanol (solution B). AZQ and its GSH conjugate were eluted using a linear gradient of 20%B to 80%B over 20 min at a flow rate of 1 ml/min and a detection wavelength of 260 nm. AZQ removal studies were performed using the same analytical method but using a detection wavelength of 344 nm. Calibration curves of peak height versus concentration of AZQ (0–20 μM) had correlation coefficients greater than 0.99.

RESULTS

Activation of AZQ in human tumor cells

HT-29 human tumor cells contained very high levels of DT-diaphorase, and the activity of this enzyme in HT-29 cell cytosol was 1948 $\pm$ 54 (n = 4) nmol/min/mg protein. Both NADH and NADPH were equally effective as electron donors. When AZQ and either NADH or NADPH were added to HT-29 cell cytosol, AZQ was removed as determined by HPLC analysis (Figure 1). Removal was NADH- or NADPH-dependent and was not observed when NADH or NADPH were mixed with AZQ in the absence of cytosol. Boiling of cytosol prior to addition of AZQ also prevented AZQ removal. Dicoumarol, an inhibitor of DT-diaphorase,[1] effectively inhibited removal of AZQ (Figure 1). These data suggested that AZQ was removed in HT-29 cells by DT-diaphorase. Since reduction of AZQ may lead to bioactivation (Scheme 1), the cytotoxicity of AZQ to HT-29 cells was measured in the presence and absence of dicoumarol. Dicoumarol was found to inhibit AZQ-induced cytotoxicity

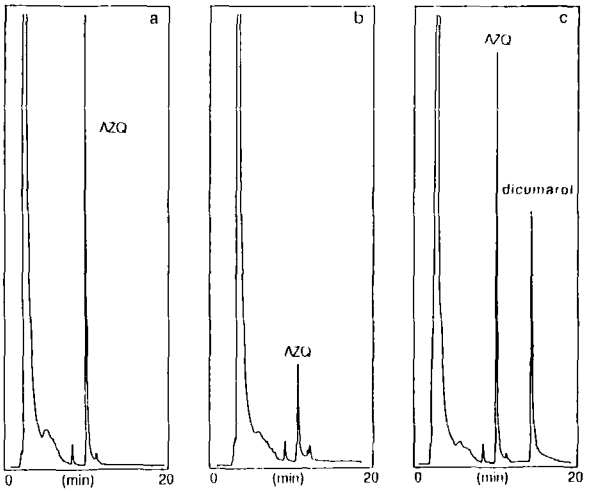


FIGURE 1 Removal of AZQ by HT-29 cell cytosol. A) AZQ (0.02 mM), NADH (0.75 mM), t = 30 min. B) AZQ (0.02 mM), NADH (0.75 mM), HT-29 cell cytosol (3 mg protein/ml), t = 30 min. C) as (b) but plus dicoumarol (0.02 mM). All reactions were performed in *Tris* 25 mM pH = 7.4, terminated by zinc precipitation, centrifuged and the supernatant used for HPLC analysis as described in Methods. Retention times were AZQ – 12.2 min and dicoumarol 17.6 min.

(Figure 2), suggesting that DT-diaphorase may be involved in activation of AZQ to a cytotoxic species.

In order to determine whether reduction of AZQ led to increased formation of a glutathione (GSH) conjugate, experiments were performed utilizing chemical reduction of AZQ. AZQ, when mixed with GSH, formed a GSH conjugate which was isolated by HPLC (Figure 3) and characterized as a conjugate by the use of ^{3}H-GSH (Figure 3, inset). Reduction of AZQ with excess sodium borohydride enhanced GSH conjugate formation. Without the addition of sodium borohydride negligible conjugate could be detected after 1 h of incubation, while in the presence of reducing

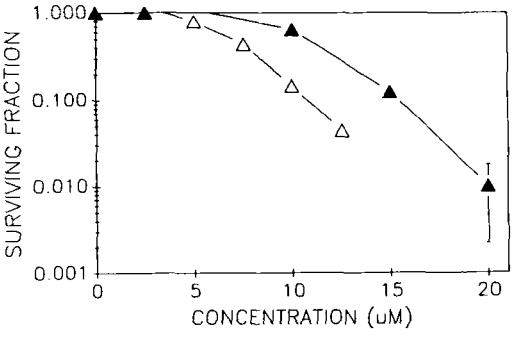


FIGURE 2 Inhibition of colony forming ability of HT-29 human colon carcinoma cells by AZQ in the presence (▲) and absence (△) of dicoumarol (0.02 mM).

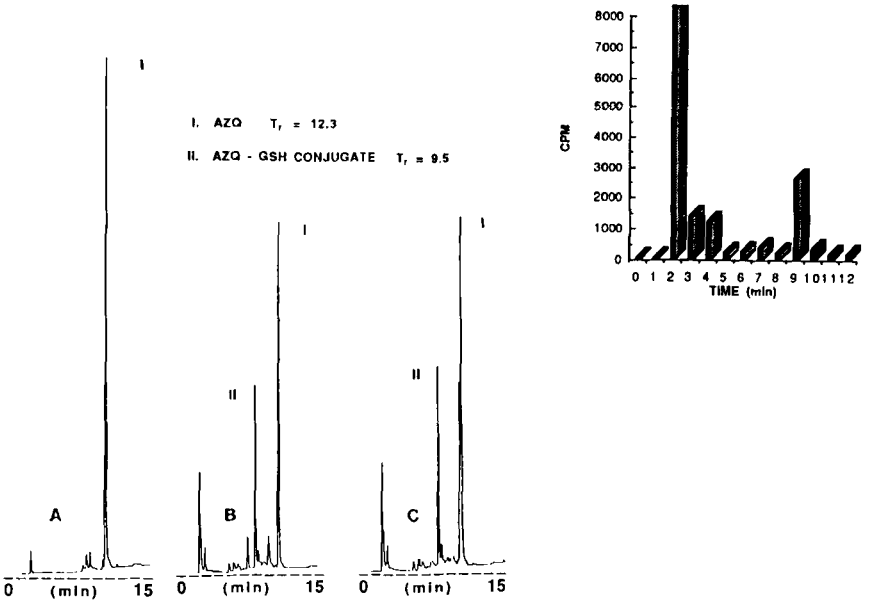


FIGURE 3 Formation of a GSH conjugate of AZQ in the presence and absence of a chemical reducing agent. A) AZQ (0.1 mM) B) AZQ (0.1 mM) plus GSH (1 mM), t = 24 h C) AZQ (0.1 mM) plus GSH (1 mM) and excess sodium borohydride t = 1 h. Inset – HPLC elution profile of ^{3}H after incubation of ^{3}H-GSH with AZQ under conditions described above in C. The ^{3}H peak eluting at 9 min corresponded with the putative AZQ-GSH conjugate peak.

agent marked conjugate formation was observed. Conjugate formation after reaction of AZQ with GSH in the absence of sodium borohydride could only be detected after prolonged incubation and the chromatogram shown in Figure 3B was obtained after 24 h.

DEACTIVATION OF HYDROQUINONE METABOLITES IN BONE MARROW STROMA

Bone marrow stroma provides structural support for immature hemopoietic cells and produces factors such as monokines and lymphokines which stimulate hemopoiesis.[24] Bone marrow stromal cell cultures consist predominantly of two cell types – macrophages and fibroblastoid stromal cells (LTF). DT-diaphorase levels were approximately fourfold higher in LTF cells than in macrophages (Figure 4). That DT-diaphorase levels were potentially important in protecting LTF cells from reactive quinones was suggested by experiments using dicoumarol. Inclusion of dicoumarol in cultures of either LTF cells or macrophages markedly increased covalent binding of ^{14}C-hydroquinone to macromolecules in LTF cells but had no significant effect on covalent binding of ^{14}C-hydroquinone in macrophages (Figure 5).

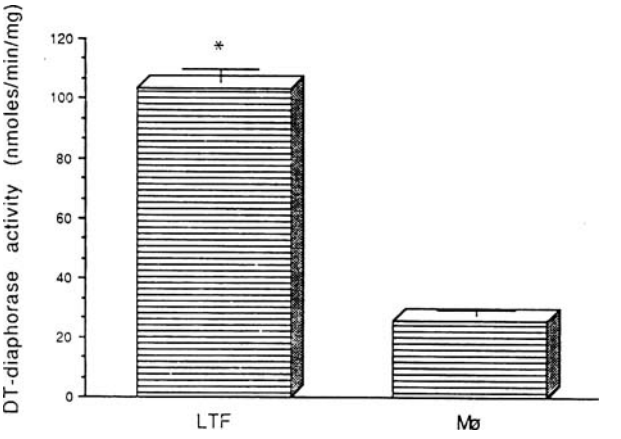


FIGURE 4 DT-diaphorase levels in macrophages and fibroblastoid stromal cells (LTF). Results represent means ± standard deviations of 5 separate determinations. ★ – Significantly different to value obtained in macrophages, $p < 0.01$ (Students t test).

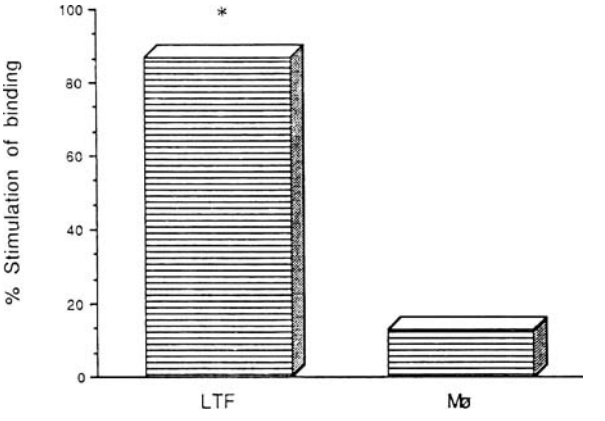


FIGURE 5 Effect of dicoumarol on covalent binding of ^{14}C-hydroquinone in macrophages and fibroblastoid stromal cells. Dicoumarol (0.02 mM) was preincubated with cells for 20 min before addition of ^{14}C-hydroquinone (0.01 mM). Incubations were performed for 3 h in serum free medium. Results shown are expressed as % increases over incubations performed in the absence of dicoumarol. Binding in the presence and absence of dicoumarol was measured in 3 separate incubations. ★ – Significantly different from incubations in the absence of dicoumarol ($p < 0.05$, Students t test).

DISCUSSION

Reductive activation of AZQ has been shown to occur in chemical systems but its role in biological systems remains poorly understood. AZQ may undergo one-electron

reduction or two-electron reduction, both of which may lead to aziridine ring opening and subsequent alkylation reactions (Scheme 1). Since both one- or two-electron reduction of AZQ may lead to bioactivation then the particular mechanism underlying activation will depend on the enzymology of the system studied. It is also relevant to point out that once the two electron reduced product is generated it will autoxidize to the semiquinone and eventually an equilibrium will be set up between quinone, hydroquinone and semiquinone.[10]

We have used a cell line which has very high DT-diaphorase levels to explore the role of this enzyme in metabolism and bioactivation of AZQ. Our data show that removal of AZQ in HT-29 cell cytosol is NADH- or NADPH-dependent and is inhibited by dicoumarol or prior boiling of the cytosol. These results are consistent with a DT-diaphorase-mediated reduction of AZQ. The observation that dicoumarol also inhibits the cytotoxicity of AZQ suggests that AZQ may be bioactivated via DT-diaphorase in HT-29 cells. Unequivocal confirmation of this must await quantitation of other indicators of AZQ bioactivation in cellular systems and studies utilizing DT-diaphorase purified from HT-29 cells. The types of cellular damage generated in cells remain to be characterized, but AZQ-induced DNA interstrand crosslinking has been observed previously in HT-29 cells.[15] Increased alkylation of cellular thiols may also be possible, since chemical reduction of AZQ in the presence of GSH led to increased formation of an AZQ-GSH conjugate. In cellular systems, however, whether extensive AZQ-GSH conjugate formation occurs remains an open question since reduction of AZQ would occur under considerably milder conditions and in the presence of competing nucleophiles.

Hydroquinone is a major metabolite of benzene and is selectively toxic at the level of the macrophage in bone marrow stroma. The fibroblastoid stromal cell (LTF) is relatively resistant to the damaging effects of hydroquinone.[24] LTF cells contained approximately fourfold higher levels of DT-diaphorase than macrophages and inhibition of this enzyme using dicoumarol markedly increased covalent binding of ^{14}C-hydroquinone to macromolecules in LTF cells. These data suggest that DT-diaphorase is critical to detoxification of hydroquinone metabolites such as 1,4-benzoquinone in LTF cells and are in agreement with the work of Smart and Zannoni,[27] who showed that added DT-diaphorase could reduce covalent binding of the phenolic metabolites of benzene in guinea pig hepatic microsomes. Thus, DT-diaphorase levels in bone marrow cells may play a role in determining the sensitivity of particular cell types to benzene metabolites.

While the role of DT-diaphorase in detoxification has been well documented there are few examples of a role for the enzyme in activation of quinones. Although preliminary, our data suggest that DT-diaphorase may catalyze bioactivation of AZQ in human tumor cells while also playing a critical role in detoxification of hydroquinone metabolites in bone marrow stromal cells. Thus, depending on chemical structure of the quinone, DT-diaphorase may be involved in activation as well as deactivation processes.

Acknowledgements

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