DIETHYLDITHIOCARBAMATE (DEDC) ENHANCES QUINONE MEDIATED OXIDATIVE STRESS CYTOTOXICITY IN ISOLATED HEPATOCYTES BY FORMING TOXIC QUINONE CONJUGATES

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Paper presented at
The First International Symposium on Sulphur Xenobiochemistry
at King's College London, September 1988.

CONTENTS

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ABBREVIATIONS

DEDC	:	diethyldithiocarbamate
DS	:	disulfiram
SOD	:	superoxide dismutase
CS_2	:	carbon disulphide
1,4-NQ	:	1,4-naphthoquinone
1,4-NQ-2-S	:	1,4-naphthoquinone-2-sulphonate
BQ	:	benzoquinone
DMSO	:	dimethyl sulphoxide
GSH	:	reduced glutathione
GSSG	:	oxidized glutathione
HEPES	:	4-(2-hydroxyethyl)-1-piperazine-ethane-suphonic acid;
HPLC	:	high performance liquid chromatography
AZ	:	azide
Enzymes	:	catalase (EC 1.11.1.6)

SUMMARY

The copper-chelating thiol drug, diethyldithiocarbamate (DEDC) had previously been used to inhibit superoxide dismutase (SOD) and enhance oxidative stress mediated cytotoxicity. Using isolated rat hepatocytes, it was confirmed that DEDC enhances oxidative stress cytotoxicity induced by 1,4-naphthoquinone (1,4-NQ) and 1,4-naphthoquinone-2-sulphonate (1,4-NQ-2S). However, equimolar concentrations of DEDC also enhances cytotoxicity induced by benzoquinone, previously shown to cause cytotoxicity as a result of alkylation and not oxidative stress. Higher DEDC concentrations on the other hand protected against benzoquinone-induced cytotoxicity. Finally, the susceptibility of hepatocytes to quinone mediated oxidative stress cytotoxicity was not enhanced if the DEDC was removed before incubating the hepatocytes with naphthoquinone or benzoquinone. Enhanced oxidative stress cytotoxicity was only observed if the DEDC was present when hepatocytes were treated with quinones. It was concluded that DEDC forms conjugates with quinones which undergo futile redox cycling in the hepatocyte and form H_2O_2 as well as increase the susceptibility of hepatocytes to H_2O_2.

I. INTRODUCTION

Diethyldithiocarbamate (DEDC) is a thiol drug which is a strong metal chelator /1,2/. Consequently, it has been found to efficiently inhibit Cu-Zn superoxide dismutase (SOD) both *in vivo* and *in vitro* /1-3/. Superoxide anion radicals have been found to damage DNA, proteins, mycoplasma, bacteria and cells in culture if not adequately removed by superoxide dismutase (SOD) which catalyses the dismutation of superoxide anion radicals /4,5/.

A marked enhancement in erythrocyte hemolysis induced by 1,4-naphthoquinone-2-sulphonate was demonstrated when cells were pretreated with DEDC /6/. Similarly, DEDC enhanced irradiation induced erythrocyte hemolysis /7/. Since DEDC inhibits SOD, it was suggested that the increase in hemolysis arose from the accumulation of superoxide anions within the cells /6,7/.

In the following it has been shown that DEDC markedly enhances the cytotoxicity of 1,4-naphthoquinone in isolated hepatocytes. This was however, not due to the inactivation of superoxide dismutase as preincubating the hepatocytes with DEDC and removing the DEDC before the addition of 1,4-naphthoquinone prevented the increase in cytotoxicity. A thiol paradox was found in the effects of DEDC on benzoquinone cytotoxicity. Equimolar DEDC concentrations enhanced the cytotoxicity of benzoquinone while excess DEDC prevented the cytotoxicity. A cytotoxic mechanism involving oxidative stress mediated by quinone-DEDC conjugates is presented.

II. MATERIALS AND METHODS

2.1 Chemical Sources

Diethyldithiocarbamate (DEDC), disulfiram (DS), carbon disulphide (CS_2), Trypan Blue, fluoro-2,4-dinitrobenzene, iodoacetic acid, GSH, GSSG, sodium azide, H_2O_2 (as a 30% solution) were obtained from Sigma Chemical Co., (St. Louis, MO). Collagenase (from *Clostridium histoliticium*), Hepes and Bovine Serum Albumin (BSA) were purchased from Boehringer-Mannheim (Montreal, Canada). KCN was obtained from the Fisher Scientific Co. All chemicals used were of the highest grade purity that was commercially available.

2.2 Animals

Adult male Sprague-Dawley rats (210-240g) fed *ad libitum* were used to prepare hepatocytes.

Isolation and Incubation of Hepatocytes

Isolated hepatocytes were prepared by collagenase perfusion of the liver as described by Moldeus et al /8/. The initial viability of hepatocytes was routinely 85%-99%. Cell viability was measured using a Neubauer chamber placed on a light microscope and counting the percentage of hepatocytes which excluded trypan blue (final concentration: 0.16% w/v). Cells at a concentration of 1×10^6 cells/ml were suspended in round bottomed flasks continuously rotating in a water bath at 37°C in Krebs-Henseleit buffer, pH7.4 supplemented with 12.5mM Hepes under a gas stream of 95% O_2 and 5% CO_2. The final incubation volume was 10ml of cells at a concentration of 10^6 cells/ml.

DEDC (100 mM) was dissolved in distilled water whereas DS and CS_2 were dissolved at a concentration of 100mM in DMSO. These were prepared immediately prior to use and added to hepatocytes after 30 minutes of preincubation.

2.3 Glutathione Assays

The total amount of GSH and GSSG in isolated hepatocytes were measured on deproteinized samples with 5% metaphosphoric acid after derivatisation with iodoacetic acid and fluoro-2,4-dinitrobenzene. Analysis was by HPLC on a μ Bondapak NH_2 column (Waters Associated, Milford, MA) /9/ using a Waters 6000A Solvent delivery system, equipped with a Model 660 solvent programmer, a WISP 710A automatic injector and a Data Module. GSH and GSSG were used as external standards.

III. RESULTS

3.1 Modulation of Quinone Induced Hepatocyte Cytotoxicity by Diethyldithiocarbamate

As shown in Fig. 1, increasing doses of DEDC (30μM-1mM) markedly increased the susceptibility of isolated hepatocytes to subtoxic doses of 1,4-naphthoquinone (1,4-NQ). Cytotoxicity increased as the concentration of DEDC increased. Similar results were obtained when 1,4-naphthoquinone-2-sulphonate was used (results not shown).

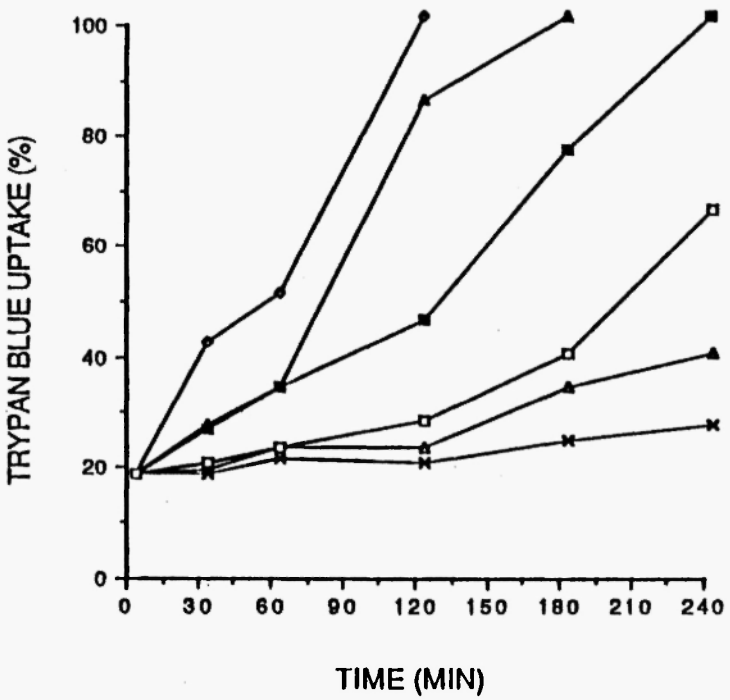

Fig. 1: DEDC 1,4-NQ induced cytotoxicity in isolated rat hepatocytes. Hepatocytes, 10^6cells/ml, were incubated without addition (x) or with 1,4-NQ (30 μM) (△) or 1,4-NQ (30 μM) plus DEDC (30 μ M) (□) or DEDC (200 μ M) (■), or DEDC (600 μ M) (▲) or DEDC (1mM) (◇). Cell toxicity was determined as the percentage of Trypan blue uptake.

As shown in Table 1 no cytotoxicity occurred when hepatocytes were incubated with DEDC only, at concentrations as high as 10mM. Carbon disulphide (10mM), a metabolite of DEDC, also did not produce any cell toxicity. The disulphide oxidation product of DEDC, disulfiram, was toxic and markedly enhanced H_2O_2 toxicity.

TABLE 1

Disulfiram Enhances the Susceptibility of Hepatocytes to Oxidative Stress

Additions	Cytotoxicity (% Trypan Blue Uptake)				
Time (mins)	30	60	120	180	240
None	13 ± 2	18 ± 2	24 ± 3	23 ± 3	30 ± 4
DEDC (1mM)	13 ± 2	19 ± 3	20 ± 2	23 ± 2	29 ± 3
DEDC (5mM)	13 ± 2	20 ± 2	25 ± 3	22 ± 2	34 ± 3
Carbon disulfide (10mM)	13 ± 3	21 ± 2	24 ± 2	20 ± 2	31 ± 3
Disulfiram (100 μM)	13 ± 2	21 ± 2	24 ± 2	20 ± 2	31 ± 3
H_2O_2 (500 μM)	14 ± 2	19 ± 1	24 ± 2	23 ± 3	32 ± 3
Disulfiram (100 μM) + H_2O_2 (500 μM)	14 ± 2	28 ± 3	38 ± 3	51 ± 4	100
Disulfiram (300 μM)	15 ± 2	39 ± 3	10 ± 3	84 ± 5	92 ± 4

Note: Hepatocytes isolated from rats were incubated (10^6 cells/ml) in Krebs-Henseleit buffer, pH 7.4, at 37°C. Cell toxicity was assessed at various times as the percentage of cells taking up Trypan blue. The values are expressed as the means of three separate experiments (± S.E.).

As shown in Table 2, upon preincubation of DEDC (1mM) with isolated hepatocytes for 30 minutes and then removal of DEDC by centrifuging the cells and resuspending them in the medium did not increase the susceptibility of hepatocytes to 1,4-NQ(25μM) or 1,4-NQ(25μM) plus DEDC(1mM).

TABLE 2

Effect of Preincubating Hepatocytes with DEDC on their Susceptibility to Naphthoquinone

Additions / Time (mins)	Cytotoxicity (% Trypan Blue Uptake) 30	90	120	180	240
None	17 ± 2	22 ± 3	22 ± 3	29 ± 4	30 ± 3
1) 30' preincub. with DEDC (1mM) & DEDC removed		22 ± 3	19 ± 3	26 ± 4	30 ± 3
2) as (1) + 1,4-NQ (25 μM)		20 ± 3	23 ± 2	26 ± 3	33 ± 4
3) as (1) + 1,4-NQ (25 μM) + DEDC (1mM)		22 ± 2	31 ± 3	95 ± 5	100

Note: Hepatocytes isolated from rats were incubated (10^6 cells/ml) in Krebs Henseleit buffer, pH 7.4, at 37°C. Cell toxicity was assessed at various times as the percentage of cells taking up Trypan blue. The values are expressed as the means of three separate experiments (± S.E.).

3.2 Effect of DEDC on Intracellular Levels of Reduced Glutathione in Isolated Hepatocytes

As shown in Fig. 2, the addition of a non-toxic dose of 1,4-naphthoquinone (10μM) to hepatocytes caused depletion of most of the GSH. GSH levels were however restored after 30 minutes of incubation. Addition of 1,4-NQ (10μM) to hepatocytes exposed to DEDC however resulted in the complete depletion of GSH. 50% of the GSH was oxidised to GSSG but was not reduced back to GSH during the course of the experiment. Thus the presence of DEDC markedly enhanced 1,4-naphthoquinone catalyzed GSH oxidation. DEDC in-

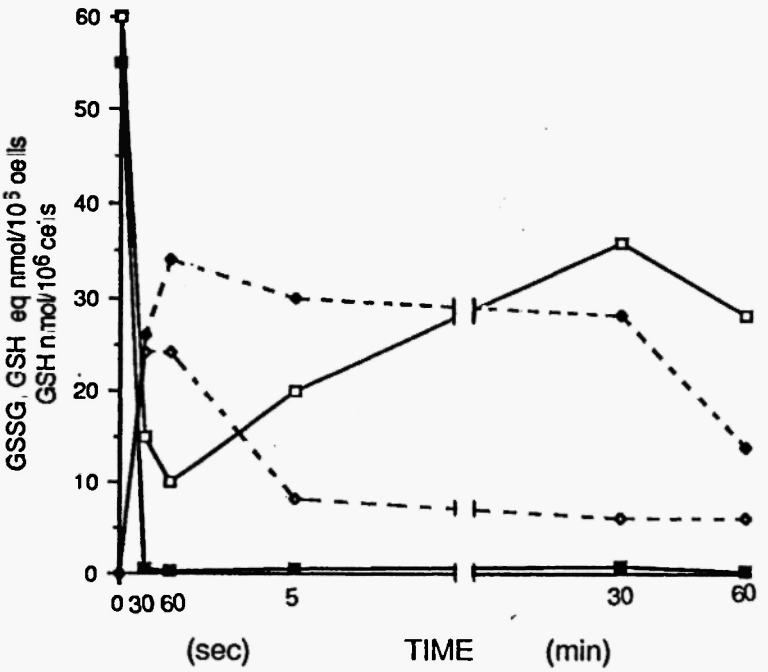

Fig. 2: Effects of 1,4-NQ and DEDC on GSH and GSSG levels. Hepatocytes (10^6cells/ml) were incubated with 1,4-NQ (10 μM) (□)-GSH, (◇) GSSG or in the presence of DEDC (200 μM) (■)-GSH, (◆)-GSSG. GSH and GSSG levels were determined by HPLC. GSSG values were corrected for the small amounts (5-10 nmol) of GSSG found in hepatocyte samples at t=o, which was located in the media.

cubated with hepatocytes had no effect on GSH levels (results not shown).

Equimolar DEDC concentrations also enhanced the cytotoxicity induced by benzoquinone. However, as shown in Fig. 3, high doses of DEDC protected isolated hepatocytes against benzoquinone-induced cytotoxicity. As shown in Fig. 4, the addition of a subtoxic dose of benzoquinone markedly depleted reduced glutathione levels in hepatocytes, which was not accompanied by GSSG formation suggesting that benzoquinone alkylated GSH. Upon addition of equimolar doses of DEDC, a greater depletion of GSH was observed. Cells treated with benzoquinone in the presence of excess DEDC resulted in a marked decrease in GSH depletion with some oxidation of GSH to GSSG

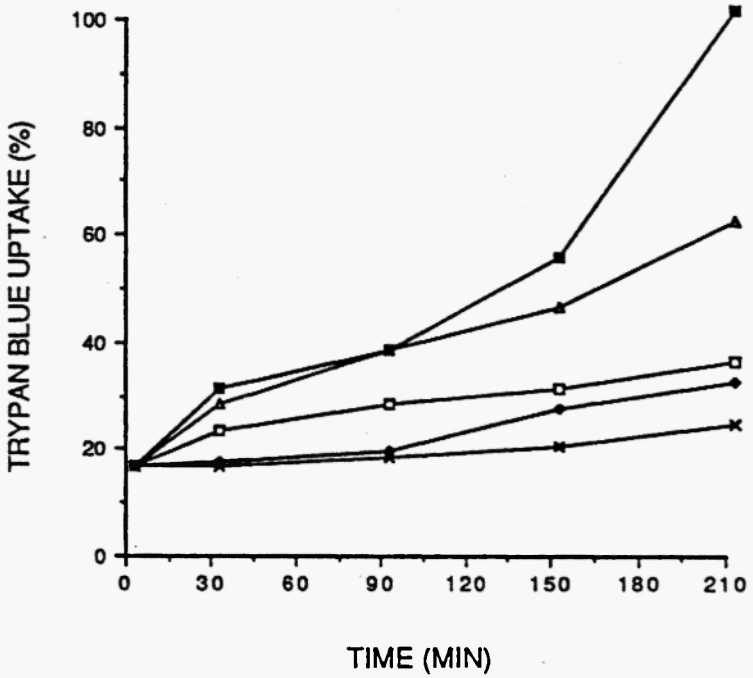

Fig. 3: Benzoquinone-induced cytotoxicity with the addition of increasing doses of DEDC in isolated rat hepatocytes. Hepatocytes (10^6cells/ml) were incubated with nothing (x), or BQ(40 μM) (◆) with DEDC (60 μM) (■), or DEDC (200 μM) (△) or DEDC (1mM) (□). Cell toxicity was determined as the percentage of Trypan blue uptake.

suggesting that DEDC prevents the alkylation of GSH and protein thiols that are probably responsible for the cytotoxicity.

IV. DISCUSSION

The present study reinvestigated the use of the copper-chelating thiol drug diethyldithiocarbamate (DEDC), a superoxide dismutase inhibitor /1,2/ in establishing the role of superoxide dismutase (SOD) in protecting the cell against the intracellular generation of superoxide radicals. Previous investigators found that DEDC markedly enhanced

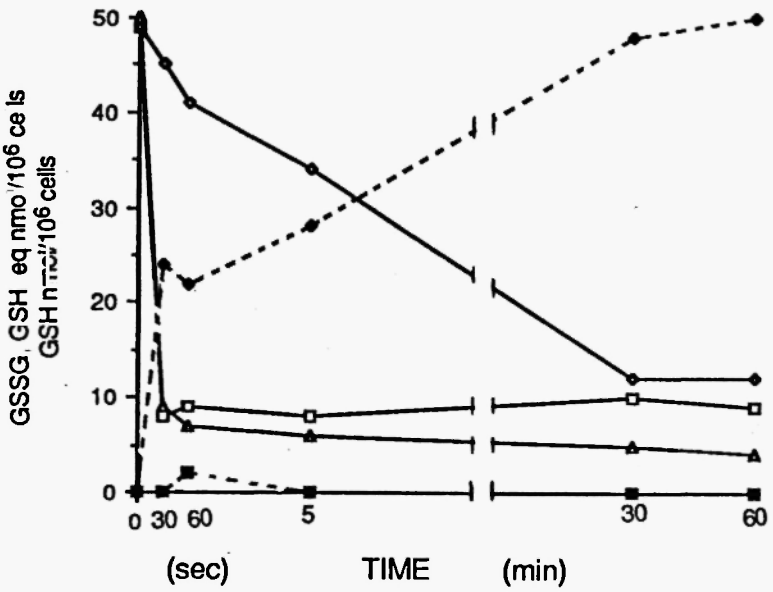

Fig. 4: Effects of BQ and DEDC on GSH and GSSG levels. Hepatocytes (10^6cells/ml) were incubated with BQ (50 μM) (□)-GSH, (■)-GSSG or in the presence of DEDC (100 μM) (△)-GSH, (■)-GSSG and DEDC (1mM) (◇)-GSH, (◆)-GSSG. GSH and GSSG levels were determined by HPLC. GSSG values were corrected for the small amounts (5-10 nmol) of GSSG found in hepatocyte samples at t=o, which was located in the media.

erythrocyte hemolysis induced by 1,4-naphthoquinone-2-sulphonate /6/.

1,4-Naphthoquinone (NQ) or 1,4-naphthoquinone-2-sulphonate cytotoxicity was also markedly enhanced when isolated hepatocytes were incubated with increasing doses of DEDC in agreement with the oxidative hemolytic results of Goldberg et al. /6/. However, U.V. spectroscopy showed that both quinones react with DEDC to form conjugates. Oxygen electrode studies showed that this reaction was accompanied by oxygen uptake and H_2O_2 formation /10/.

Disulfiram, the oxidised metabolite of DEDC, was formed with high DEDC concentration and NQ as demonstrated by HPLC chromatography

/10/. subsequently, disulfiram was found to markedly enhance the susceptibility of isolated hepatocytes to H_2O_2 whereas carbon disulphide, another metabolite of DEDC /11/ was not toxic. This suggests that disulfiram may play a role in compromising the hepatocyte and contribute to the increased cytotoxicity when high doses of DEDC are added to NQ treated cells. The following equations could explain the formation of disulfiram when excess DEDC is added to NQ. Reaction (1) outlines a two electron oxidation of DEDC to disulfiram in which the conjugate is reduced to the hydroquinone conjugate (NHQ · DEDC), which autoxidises (reaction (2)) to reform the quinone. Reactions (3) and (5) outline two one electron oxidation steps in which DEDC reduced the conjugate to an autoxidisable semiquinone conjugate (NSQ · DEDC). Disulfiram could then form by the dimerisation of DEDC thiyl radicals (reaction (4)).

$$NQ\cdot DEDC \xrightarrow{+\,2\text{-}DEDC} NHQ\cdot DEDC + DS \qquad (1)$$

$$NHQ\cdot DEDC + O_2 \longrightarrow NQ\cdot DEDC + H_2O_2 \qquad (2)$$

$$NQ\cdot DEDC + DEDC \longrightarrow NSQ\ DEDC + DEDC^{\bullet} \qquad (3)$$

$$2DEDC^{\bullet} \longrightarrow DS \qquad (4)$$

$$NSQ\cdot DEDC + O_2 \longrightarrow NQ\cdot DEDC + O_2^{\bullet-} \qquad (5)$$

A scheme to explain the mechanism of DEDC enhanced naphthoquinone cytotoxicity is described in Fig. 5.

Mammalian cells are normally well equipped with enzymic defence mechanisms against H_2O_2 and active oxygen species so that these cells need to be compromised by impairing their defence mechanisms before the cells become susceptible. Disulfiram has been found to inhibit various enzymes, such as glucose-6-phosphate dehydrogenase /12/ which is necessary for the production of NAD(P)H required by glutathione reductase to maintain GSH in the reduced form. This could explain why hepatocytes were susceptible to H_2O_2 in the presence of disulfiram. It is therefore postulated that disulfiram and/or the NQ:DEDC conjugate formed by the reaction between NQ and DEDC compromises the hepatocyte, so that its defence against cytotoxic

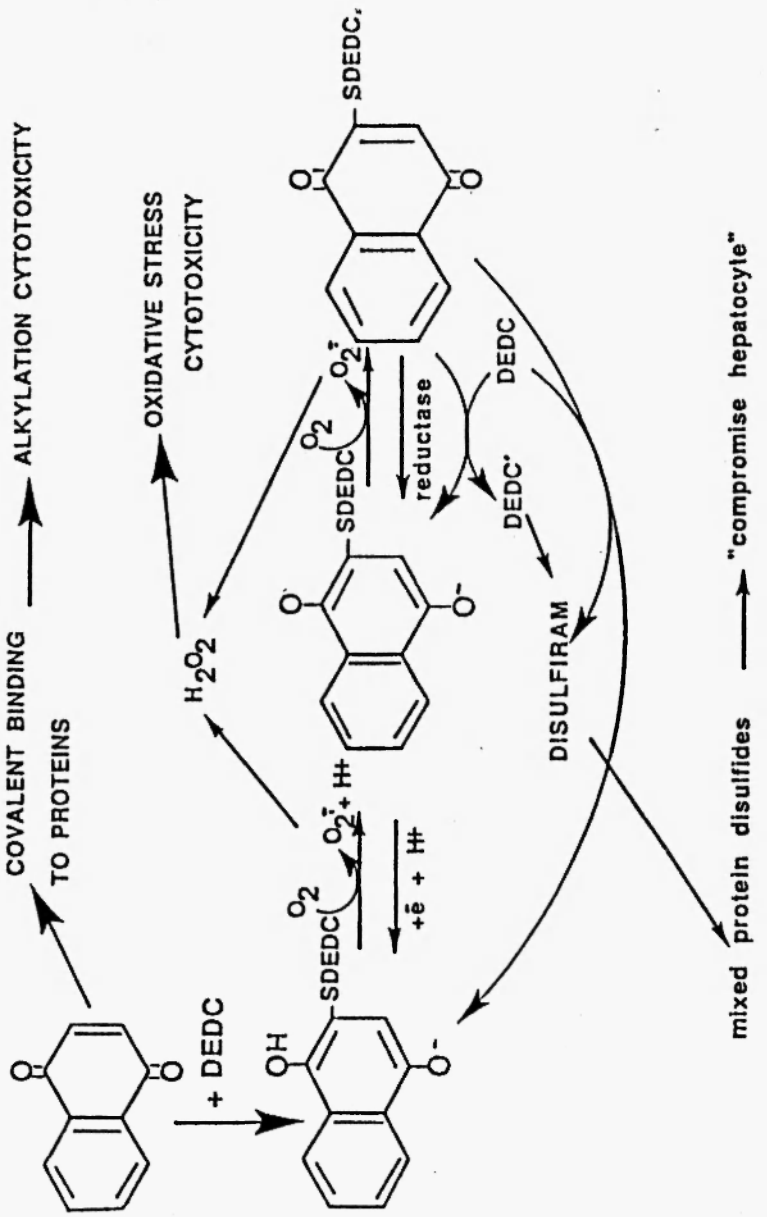

Fig. 5: Naphthoquinone induced cytotoxicity in isolated hepatocytes.

H_2O_2 generated by the redox cycling of NQ and the NQ:DEDC conjugate /10/ is impaired.

The effect of DEDC on the cytotoxicity of benzoquinone was highly dose-dependent. Low or equimolar doses of DEDC markedly enhanced benzoquinone cytotoxicity, while excess DEDC protected the cells. Benzoquinone was previously shown to be cytotoxic to hepatocytes as a result of the alkylation of proteins and no evidence of oxidative stress was found /13/. Upon addition of an equimolar concentration of DEDC to the benzoquinone, GSH depletion still occurred suggesting that the benzoquinone:DEDC conjugate can still alkylate GSH and protein thiols. The GSH oxidation that occurs indicates that these conjugates undergo futile redox cycling in the hepatocyte resulting in harmless H_2O_2 formation /4/. At low DEDC concentrations, benzoquinone induced cytotoxicity is enhanced because the hepatocyte is compromised as a result of protein alkylation by the benzoquinone and is susceptible to H_2O_2 generated by redox cycling of the benzoquinone:DEDC conjugate. At higher DEDC concentrations GSH depletion was inhibited and cytotoxicity prevented. These results suggest that high DEDC concentrations prevents the benzoquinone from alkylating protein thiols by forming nontoxic benzoquinone:DEDC conjugates.

When one equimolar equivalent of DEDC was added to benzoquinone, the benzoquinone disappeared and was converted to a conjugate (HQ·DEDC) which was collected by repetitive HPLC. The FAB mass spectrum of the isolated conjugate gave m/z 280 (M+Na; relative intensity, 25%), 258 (M+1; relatve intensity, 100%), 256 (M+1-2H; S; relative intensity, 35%) and 116 ($C\text{-}N(C_2H_5)_2$; relative intensity, 75%) consistent with a monosubstituted conjugate /10/. When DEDC was added to benzoquinone, oxygen uptake also occurred and H_2O_2 was formed. The following reactions explain this:

$$BQ + DEDC \longrightarrow HQ\cdot DEDC \qquad (6)$$

$$HQ\cdot DEDC + O_2 \longrightarrow SQ\cdot DEDC + O_2^{\cdot-} \qquad (7)$$

$$SQ\cdot DEDC + O_2 \longrightarrow Q\cdot DEDC + O_2^{\cdot-} \qquad (8)$$

$$2HO_2^{\cdot-} \longrightarrow H_2O_2 + O_2 \qquad (9)$$

A scheme to explain the effects of DEDC on benzoquinone cytotoxicity is described in Fig. 6.

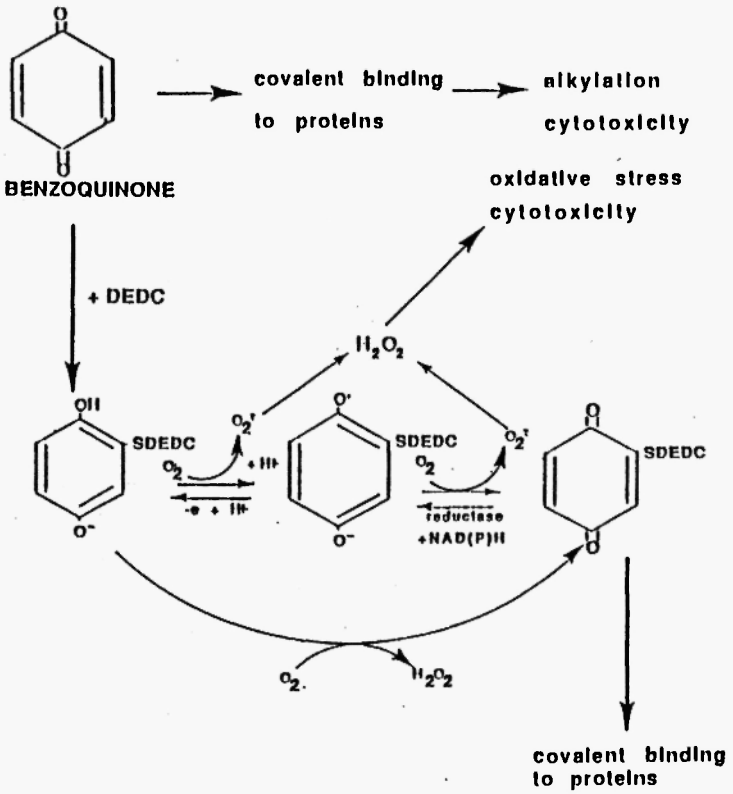

Fig. 6: Benzoquinone induced cytotoxicity in isolated hepatocytes.

DEDC has been found to irreversibly inactivate superoxide dismutase by chelating the Cu(II) /3/. DEDC did not enhance naphthoquinone mediated exidative stress cytotoxicity by inactivating superoxide dismutase as hepatocytes preincubated with DEDC to inactivate superoxide dismutase were not more susceptible to naphthoquinone mediated oxidative stress if DEDC was removed beforehand.

DEDC therefore modulates quinone mediated hepatocyte cytotoxicity as a result of forming DEDC-quinone conjugates which undergo futile redox cycling and form cytotoxic H_2O_2.

REFERENCES

1. Heikkila, R.E., Cabbat, F.S. and Cohen, G. *In vivo* inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J. Biol. Chem.* 1976; **251**:2182-2185.
2. Sinet, P.M., Garber, P. and Jerome, H. H_2O_2 production, modification of the glutathione status and methemoglobin formation in red blood cells exposed to diethyldithiocarbamete *in vitro*. *Biochem. Pharmacol.* 1982; **31**:521-525
3. Misra, H.P. Reaction of copper-zinc superoxide dismutase with diethyldithiocarbamate. *J. Biol. Chem.* 1979; **254(22)**:11623-11628.
4. Cohen, G.M. and d'Arcy Doherty, M. Free radical mediated cell toxicity by redox cycling chemicals. *Br. J. Cancer.* 1987; **55, suppl. VII**:46-52.
5. Halliwell, B. and Gutteridge, J.M.C. In: Free Radicals in Biology and Medicine, Oxford: Clarendon Press, 1985.
6. Goldberg, B. and Stern, A. Superoxide anion as a mediator of drug-induced oxidative hemolysis. *J. Biol. Chem.* 1976; **251**:6468-6470.
7. Westman, G. and Marklund, S.L. Diethyldithiocarbamate, a superoxide dismutase inhibitor decreases the radioresistance of Chinese hamster cells *Rad. Res.* 1980; **83**:303-311.
8. Moldeus, P., Hogberg, J. and Orrenius, S. Isolation and use of liver cells. In: Hoffee, P.A. and Jones, M.E., eds., Methods in Enzymology, New York: Academic Press, 1978; Vol.51:60-71.
9. Reed, D.J., Babson, J.R., Beatty, B.W., Brodie, A.E., Ellis, W.W. and Potter D.N. High-performance liquid chromatography analysis of nanomoles levels of glutathione, glutathione sulfide, and related thiols and disulfides. *Analyt. Biochem.* 1980; **106**:55-62.
10. Lauriault, V.V.M., Wong, W.C., McGirr, L.G. and O'Brien, P.J. *(submitted for publication).*
11. Eneanya, D.I., Bianchine, J.R., Duran, D.O. and Andersen, B.D. The actions and metabolic fate of disulfiram. *Ann. Rev. Pharmacol. Toxicol.* 1981; **21**:575-596.
12. Nygaard, A.P. and Sumner, J.H. D-Glyceraldehyde-3-phosphate dehydrogenase; a comparison with liver aldehyde dehydrogenase. *Arch. Biochem. Biophys.* 1952; **39**:119-128.
13. Rossi, L., Moore, G.A., Orrenius, S. and O'Brien, P.J. Quinone toxicity in hepatocytes without oxidative stress. *Arch. Biochem. Biophys.* 1986; **251**:25-35.
14. Rossi, L., Silva, J.M., McGirr, L.G. and O'Brien, P.J. Nitrofurantoin-mediated oxidative stress cytotoxicity in isolated rat hepatocytes. *Biochem. Pharmacol.* 1988; **37**:3109-3117.