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INHIBITION OF THIOREDOXIN REDUCTASE (E.C. 1.6.4.5.) BY ANTITUMOR QUINONES

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Thioredoxin reductase (TR) is a widely distributed flavoenzyme that provides reduced thioredoxin, a dithiol hydrogen donor for protein disulfide reduction and for the reduction of ribonucleotides to deoxyribonucleotides, the first unique step of DNA synthesis. Antitumor quinones were found to exhibit time- and concentration-dependent inhibition of purified rat liver TR that requires the presence of NADPH. Diaziquone initially shows competitive inhibition of the enzyme with 5,5'-dithiobis 2-nitrobenzoic acid as substrate with a K_i of 7.5 μM, which becomes non-competitive after 1 hour incubation with NADPH with a K_i of 0.5 μM. Doxorubicin shows non-competitive inhibition both initially and after 1 hr incubation with NADPH, with K_i values of 10 μM and 0.5 μM, respectively. Electron spin resonance spectroscopy showed the formation of semiquinone free radicals by TR incubated under anaerobic conditions with doxorubicin or diaziquone and NADPH. Redox cycling and formation of oxygen radicals does not play a major role in the inhibition of TR by antitumor quinones as shown by the minor effect on inhibition of removing O_2, and the lack of effect of superoxide dismutase and catalase. Diaziquone causes time- and concentration-dependent inhibition of TR activity in intact A204 human rhabdomyosarcoma cells that is associated with growth inhibition. The results suggest that inhibition of TR by antitumor quinones could contribute to their growth inhibitory properties.

KEY WORDS: Thioredoxin reductase, doxorubicin, diaziquone, antitumor, cell growth.

INTRODUCTION

Thioredoxin reductase (E.C. 1.6.4.5.) is a widely distributed NADPH-dependent flavoenzyme.[1,2] Its natural substrate is thioredoxin, a protein which contains a reducible cystine disulfide. Reduced thioredoxin is a dithiol hydrogen donor for ribonucleotide reductase.[3] It is also a powerful protein disulfide reductase[4] and a hydrogen donor for enzymes catalyzing the reduction of sulfoxides and sulfate.[1]

Antitumor quinones are among the most active of the currently available anticancer drugs but their mechanism of action is unresolved.[5] Antitumor quinones are known to be metabolized by flavoproteins such as NADPH-cytochrome P-450 reductase (E.C. 1.6.2.2) to give semiquinone free radicals that can undergo subsequent oxidation in air with the formation of oxygen radicals[6,7,8] A feature of antitumor quinones not usually seen with simple quinones is the ability to form alkylating species that can bind to DNA and other cellular macromolecules.[5] As part of our continuing efforts to understand the mechanism of action of antitumor quinones we have studied

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the metabolism of selected antitumor quinones by thioredoxin reductase and shown them to have the characteristics of suicide substrate inhibitors. Inhibition of thioredoxin reductase activity by the antitumor quinones was also seen in intact tumor cells and was associated with growth inhibition.

METHODS

Thioredoxin reductase was purified from male Sprague Dawley rat liver by a modification of the method of Luthman and Holmgren.[9] After 2′,5′-ADP-Sepharose affinity chromatography Cibacron Blue Sepharose CL-6B (Pharmacia, Inc., Piscataway, NJ) affinity chromatography was employed instead of ω-aminohexyl agarose chromatography. A 7,300-fold purification of thioredoxin reductase with a yield of 23% was achieved, with a final specific activity at 23°C of 37.4 μmol 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) reduced/min/mg enzyme protein. The incubation mixture for the assay of thioredoxin reductase activity contained 100 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.2 mg/ml bovine serum albumin, 0.5 to 1 μg thioredoxin reductase, 0.2 mM NADPH, 1 mM DTNB and up to 10^{-4} M of antitumor quinones. DTNB and the antitumor quinones were dissolved in DMSO as stock solutions. Reduction of DTNB was measured at 412 nm. Antitumor quinones were added at the same time as DTNB, or up to 60 min before DTNB together with NADPH. Some reactions were conducted in sealed cuvettes after oxygen had been removed by thoroughly purging the solution with N_2. Electron spin resonance spectroscopy (esr) employed a Bruker model ER200 spectrometer.

Measurement of thioredoxin reductase activity in sonicates of A204 human rhabdomyosarcoma cells exposed to antitumor quinones employed a modified method of Luthman and Holmgren.[9] A two-stage procedure was necessary to prevent DTNB being reduced by other reductases in the cell sonicates. Insulin was used as the substrate for reduction by thioredoxin and thioredoxin reductase in the cell sonicates. This was followed by reduction of DTNB by the reduced insulin in the presence of 6 M guanidine hydrochloride. A background correction for reduction of DTNB by endogenous reduced thiols was made. A204 human rhabdomyosarcoma cells were incubated with antitumor quinones for different periods of time up to 24 hr, washed and the thioredoxin reductase activity was measured. Cell growth was measured as colony formation 7 days later employing the soft agarose assay[10] with an aliquot of the same cells exposed to antitumor quinones.

TABLE I

Inhibition of DTNB Metabolism by Thioredoxin Reductase Caused by Anticancer Quinones. Metabolism of DTNB by thioredoxin reductase was determined as described in the text. Antitumor quinones were incubated with enzyme and NADPH for 0 or 7 min before adding DTNB

Quinone	Without preincubation 10^{-4} M % inhibition	Preincubated for 7 min	
		10^{-4} M % inhibition	10^{-5} M % inhibition
Doxorubicin	0	80	33
Diaziquone	24	94	94
Menadione	23	85	29
Mitomycin C	13	29	ND
Dichloroallyl Lawsone	0	35	ND

ND = not determined.

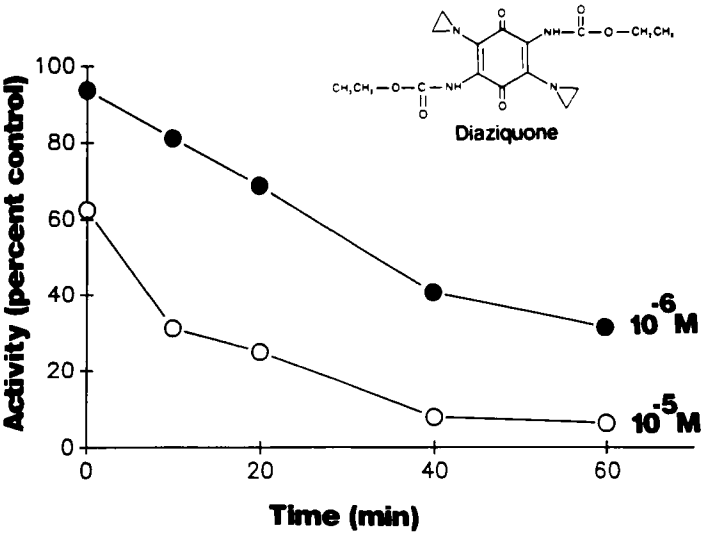

FIGURE 1 Time course of the inhibition of DTNB metabolism by thioredoxin reductase caused by diaziquone. Incubations contained 0.2 mM NADPH, 1 μg/ml thioredoxin reductase and diaziquone at the concentrations shown for various times before addition of 1 mM DTNB.

RESULTS

Incubation of a number of antitumor quinones with thioredoxin reductase and NADPH showed some inhibition of the metabolism of DTNB when DTNB was added at the same time as the antitumor quinone (Table 1). More pronounced inhibition of DTNB metabolism was seen when DTNB was added several minutes

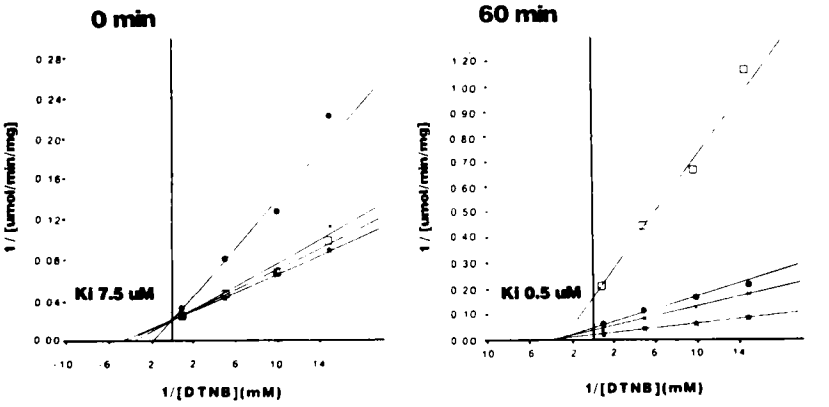

FIGURE 2 Kinetics of the inhibition of DTNB metabolism caused by diaziquone. Left panel, DTNB added together with NADPH and diaziquone at concentrations of (★) 0 mM; (□) 10^{-6} M; (×) 2×10^{-6} M and (●) 10^{-5} M. Right panel DTNB added 60 min after NADPH and diaziquone at concentrations of (★) 0 mM; (×) 10^{-7} M; (●) 10^{-6} M and (□) 10^{-5} M. Continuous lines are computer generated fits to the data (Enzfitter, Biosoft).

TABLE II
Inhibition of DTNB Metabolism by Thioredoxin Reductase Under Aerobic and Anaerobic Conditions. Metabolism of DTNB was measured after 60 min preincubation with the antitumor quinone and NADPH under either aerobic or anaerobic conditions. Values are the percent inhibition compared to a control without antitumor quinone and are the mean ± S.E. of 3 determinations

Quinone	Air % inhibition	N_2 % inhibition
Doxorubicin		
10^{-5} M	88 ± 1	71 ± 4[a]
10^{-6} M	77 ± 4	47 ± 6[a]
Diaziquone		
10^{-5} M	88 ± 2	73 ± 4[a]
10^{-6} M	52 ± 0	40 ± 1[a]

[a] $P < 0.05$ compared to value in air.

after the antitumor quinone. There was no inhibition of enzyme activity by the antitumor quinones when the preincubation was conducted without NADPH (results not shown). A time course of the inhibition of DTNB metabolism by preincubation of thioredoxin reductase with diaziquone and NADPH is shown in Figure 1. The amount of NADPH in the assay was not limiting for DTNB metabolism even after 1 hr incubation.

Kinetic studies showed that diaziquone initially exhibited competitive inhibition of DTNB metabolism by thioredoxin reductase but the inhibition became non-competitive after 1 hr preincubation (in the presence of NADPH) with a decrease in K_i from 7.5 μM to 0.5 μM (Figure 2). Doxorubicin also showed a decrease in K_i after 1 hr preincubation from 10 μM to 0.5 μM, but the inhibition was initially non-competitive as well as after 1 hr.

Studies were conducted to determine whether using anaerobic conditions had an effect on the inhibition of thioredoxin reductase by the antitumor quinones (Table 2). There was only a small reduction in the inhibition suggesting that oxygen radicals were not the major species responsible for inhibition. The use of superoxide dismutase and catalase to scavenge superoxide anion radical and hydrogen peroxide had no effect on the inhibition of enzyme activity (Table 3).

ESR studies showed the formation of a semiquinone free radical signal when doxorubicin was incubated with thioredoxin reductase and NADPH under anaerobic

TABLE III
Effect of Radical Scavengers on Inhibition of DTNB Metabolism Caused by Antitumor Quinones. Metabolism of DTNB was measured after 60 min preincubation with the antitumor quinone at 10^{-5} M and NADPH. The radical scavengers used were superoxide dismutase 10 μg/ml and catalase 30 μg/ml. Values are the percent inhibition compared to a control value without antitumor quinone or scavenger and are the mean ± S.E. of 3 determinations.

	No scavenger % inhibition	SOD % inhibition	Catalase % inhibition	SOD + catalase % inhibition
Doxorubicin	90 ± 1	94 ± 19	89 ± 0	92 ± 0[a]
Diaziquone	67 ± 1	71 ± 2	63 ± 1	68 ± 2

[a] $P < 0.05$ compared to value with no scavengers.

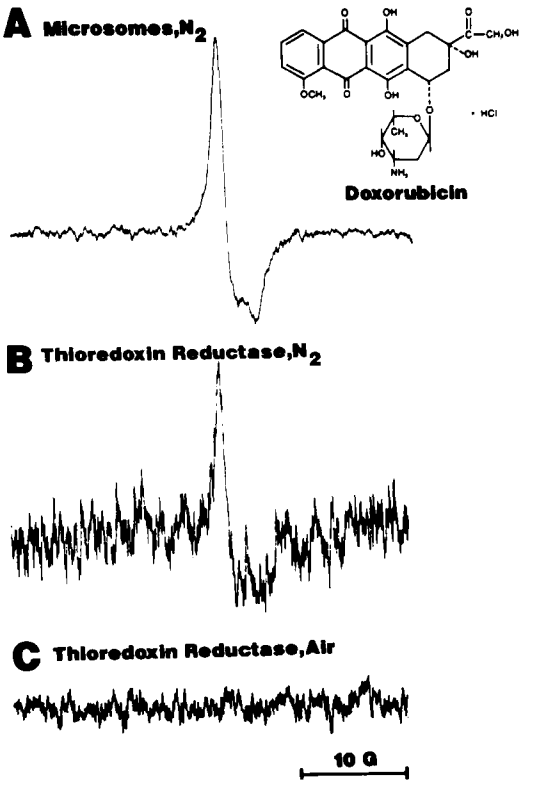

FIGURE 3 ESR spectra showing of doxorubicin semiquinone radical by thioredoxin reductase. A, 3 mM doxorubicin incubated with rat hepatic microsomes 1 mg protein/ml and 1 mM NADPH for 5 min under N_2; B, 3 mM doxorubicin incubated with thioredoxin reductase 50 μg/ml and 1 mM NADPH for 10 min under N_2; C, 3 mM doxorubicin incubated with thioredoxin reductase 50 μg/ml and 1 mM NADPH for 10 min in the presence of air. The instrument parameters were: 50 gauss/3 min sweep time, time constant 0.6 sec, modulation frequency 100 kHz, modulation amplitude 0.8 gauss, temperature 25°C, gain 8 $\times$ 10^4.

conditions (Figure 3). The signal was not seen under aerobic conditions. Diaziquone incubated with thioredoxin reductase and NADPH also gave an esr semiquinone radical signal under anaerobic conditions but in this instance the signal was not completely abolished in the presence of air (results not shown).

Incubation of A204 human rhabdomyosarcoma cells with diaziquone for up to 24 hr resulted in time- and concentration dependent inhibition of cellular thioredoxin reductase activity and inhibition of cell growth measured by colony formation in soft agarose (Figure 4).

DISCUSSION

Antitumor quinones have been shown to produce a time- and concentration-dependent inhibition of rat liver thioredoxin reductase. The fact that NADPH was necessary for the inhibition to occur and, with diaziquone at least, there was a change in

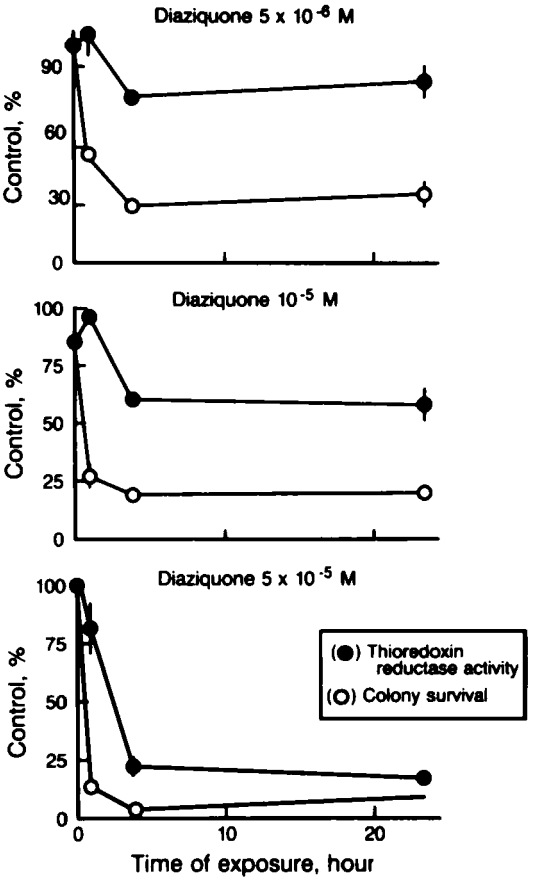

FIGURE 4 Inhibition of thioredoxin reductase activity in A204 tumor cells and cell growth inhibition by exposure for diaziquone for various times.

the characteristics of the inhibition from initially competitive to non-competitive after 1 hr incubation, suggests that metabolism of the antitumor quinone is necessary for enzyme inhibition.

Inhibition of flavoenzymes by antitumor quinones has been previously reported for NADPH-cytochrome P-450 reductase by ametantrone, a *bis*(substituted aminoalkylamino) anthraquinone.[11] In this case inhibition was explained by binding of the bis(substituted aminoalkylamino)anthraquinone to flavin prosthetic groups in the enzyme.[12]

Antitumor quinones are metabolized by a number of flavoenzymes to semiquinone free radicals.[5] In the presence of oxygen there is redox cycling of the semiquinone free radical to form oxygen radicals.[7,8] Metabolism of the antitumor quinones by thioredoxin reductase led to the formation of a semiquinone free radical. Oxygen radicals did not appear to play a major role in the inhibition of thioredoxin reductase by doxorubicin or diaziquone, as shown by the relatively small effect of conducting the incubations under N_2 when oxygen radicals could not be formed. The use of

superoxide dismutase and catalase as scavengers of oxygen radicals had no effect on the inhibition of the enzyme, although these proteins might have difficulty penetrating to the site of antitumor quinone metabolism by thioredoxin reductase. Reduction of doxorubicin[13] and diaziquone[14] has been shown to lead to the formation of alkylating species. Preliminary studies in our laboratory have shown covalent binding of diaziquone to thioredoxin reductase. We propose that metabolism of some antitumor quinones by thioredoxin reductase leads to the formation of a reactive intermediate, that may be the semiquinone free radical, that binds to and inactivates the enzyme.

The consequences of inhibition of thioredoxin reductase by antitumor quinones is not known. We found an association between inhibition of thioredoxin reductase activity by diaziquone in intact tumor cells and growth inhibition measured in the same cells several days later. Thioredoxin is an important cofactor for ribonucleotide reductase[3] which in mammalian cells, unlike bacteria, cannot use the glutathione/glutaredoxin system as a source of reducing equivalents.[15] Inhibition of thioredoxin reductase might, therefore, affect DNA synthesis. Thioredoxin is a powerful protein disulfide reductase[4] and has been reported to affect glucocorticoid receptor activity.[16] There could also be an effect of inhibition of thioredoxin reductase on cell growth through such mechanisms. It is possible, therefore, that inhibition of thioredoxin reductase could contribute to the growth inhibitory activity of antitumor quinones.

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