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# MECHANISM-BASED INHIBITION OF THIOREDOXIN REDUCTASE BY ANTITUMOR QUINOID COMPOUNDS

BEY-LIING MAU and GARTH POWIS*
Department of Pharmacology, Mayo Clinic and Foundation, Rochester, MN 55905, U.S.A.

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**Abstract**—Quinoids underto metabolism by a number of flavoenzymes. Reactive species formed during the metabolism of some quinoids might be anticipated to inhibit flavoenzyme activity. Several quinoids have been tested for their ability to inhibit rat liver thioredoxin reductase (TR). The antitumor quinones diaziquone and doxorubicin, and the quinoneimine 2,6-dichloroindophenol, were found to be inhibitors of the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) by TR. The inhibition was most marked after incubation of the quinoid with NADPH and the enzyme for 60 min before adding DTNB, with $K_i$ values of 0.5 μM for diaziquone, 0.5 μM for doxorubicin, and 0.07 μM for 2,6-dichloroindophenol. The three quinoids all produced a time-dependent and first order loss of TR activity. There was formation of electron spin resonance-detectable semiquinoid free radicals upon incubation of diaziquone, doxorubicin and 2,6-dichloroindophenol with TR and NADPH under anaerobic conditions. Oxygen radicals formed by redox cycling of the quinoids did not make a major contribution to the inhibition of TR by the quinoids, as shown by the absence of significant reversal of the inhibition by anaerobic incubation conditions and the lack of effect of the oxygen radical scavengers dimethyl sulfoxide, superoxide dismutase and catalase. It was not possible to demonstrate NADPH-dependent covalent binding of radiolabeled diaziquone or doxorubicin to the TR apoprotein. It is possible that the quinoids bind noncovalently to the enzyme apoprotein, or bind to the FAD prosthetic group. The results of the study suggest that some antitumor quinoids are mechanism-based inhibitors of TR showing metabolism- and time-dependent irreversible inhibition of enzyme activity.

Quinones are among the most widely used and effective drugs for the treatment of human cancer (reviewed in Ref. 1). The antitumor quinones have a diverse range of chemical structures and exhibit many different properties including the ability to bind to and degrade DNA [2, 3], inhibition of nuclear DNA topoisomerase II [4, 5], membrane-altering properties [6, 7] and the generation of free radicals [8, 9]. None of these mechanisms by themselves has proven sufficient to explain the wide spectrum of activity of the antitumor quinones [10, 11].

The metabolism of antitumor quinones appears to be important for their biological activity (reviewed in Ref. 1). Antitumor quinones are known to undergo reduction by a number of flavoenzymes, including NADPH–cytochrome P450 reductase, NAD(P)H-(quinone acceptor) oxidoreductase (DT-diaphorase), and xanthine oxidase [12–14]. The reduction of antitumor quinones gives rise to reactive species that can bind covalently to DNA [15, 16] or react with molecular oxygen giving rise to reactive oxygen species that can cause DNA degradation [3, 17–19]. Both mechanisms have been suggested to contribute to the antitumor activity of the antitumor quinones [19–23]. Another possibility we now consider is that flavoenzymes are themselves targets for inactivation by reactive species formed during the reductive metabolism of antitumor quinones. If the flavoenzyme inhibited is important for cell proliferation, this might contribute to the growth inhibitory activity of the antitumor quinones.

For this investigation we chose to study the flavoprotein thioredoxin reductase (TR†; EC 1.6.4.5). TR is a flavoenzyme found in almost all living cells [24]. The natural substrate for mammalian TR is a small, redox-active protein, thioredoxin, which has two half-cystine residues at its active center [24]. Thioredoxin can exist either in the dithiol-reduced form or in the oxidized disulfide form. Oxidized thioredoxin is reduced by TR employing NADPH as a cofactor, giving reduced thioredoxin which participates in many thiol-dependent, cellular processes. Thioredoxin acts as an electron donor for a number of enzymes including ribonucleotide reductase, which catalyzes the first unique step of DNA synthesis [24], vitamin K epoxide reductase [25], methionine sulfoxide reductase [26] and protein disulfide isomerase [27]. Reduced thioredoxin also acts as a cellular free radical scavenger [28] and as an endogenous activating factor of the cytosolic glucocorticoid receptor [29]. Thioredoxin domains have been identified in the hormones follitropin and luteotropin [30], in protein disulfide isomerase [31], in phosphoinositide phospholipase C-α [32] and in the autocrine growth factor, adult T-cell leukemia-

* Corresponding author: Dr. Garth Powis, Arizona Cancer Center, University of Arizona College of Medicine, 1501 N. Campbell Avenue, Tucson, AZ 85724. Tel. (602) 626-6408; FAX (602) 626-2284.

† Abbreviations: TR, thioredoxin reductase; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; and SOD, superoxide dismutase.

derived factor (ADF) [33]. A decrease in the levels of reduced thioredoxin would be expected to have important consequences for cellular function.

We report here the irreversible metabolism and time-dependent inactivation of mammalian TR by some antitumor quinoids. A preliminary report of a part of this work has appeared [34].

## MATERIALS AND METHODS

*Chemicals and enzymes.* Doxorubicin, mitomycin C, 2,6-dichloroindophenol, streptonigrin, actinomycin D, 2-methyl-1,4-naphthoquinone (menadione), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), superoxide dismutase (SOD), catalase, FAD, and NADPH were purchased from the Sigma Chemical Co. (St. Louis, MO). Diaziquone, mitoxantrone, [ring-$^{14}$C]diaziquone (13.6 mCi/mmol), and [14-$^{14}$C]-doxorubicin (12.2 mCi/mmol) were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD).

*Enzyme purification.* TR was partially purified from the livers of nonfasted, male Sprague–Dawley rats by a modification of the method of Luthman and Holmgren [35]. We found that the ammonium sulfate precipitation described by these authors did not increase the specific activity of the enzyme and the step was omitted. The ω-aminohexyl agarose affinity column described by Luthman and Holmgren [35] was replaced by a Cibacron Blue-Sepharose CL-6B affinity column (Pharmacia, Piscataway, NJ). The final specific activity of a typical TR preparation was 37.4 μmol DTNB reduced/min/mg protein at room temperature (see assay conditions below). This represented a 7343-fold purification of enzyme activity from rat liver and an overall yield of 23%, which are similar to the values reported by Luthman and Holmgren [35]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the final preparation gave a single, Coomassie Blue staining band at 58 kDa which corresponds to the reported molecular weight of the rat liver TR subunit [35].

*Assays.* TR activity was measured by the reduction of DTNB to thionitrobenzoic acid at 412 nm [36]. The assay was conducted at room temperature. The incubation mixture contained 0.5 to 1 μg TR protein in 1 mL of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.2 mg/mL bovine serum albumin and 0.2 mM NADPH. The reaction was initiated by the addition of 1 mM DTNB dissolved in dimethyl sulfoxide as a 100× stock solution. The quinoids, at concentrations of $10^{-8}$ to $10^{-5}$ M, were added immediately before the DTNB or were preincubated with the TR and NADPH for up to 60 min before adding the DTNB. Diaziquone, doxorubicin, menadione, actinomycin D and streptonigrin were added as 100× stock solutions dissolved in dimethyl sulfoxide, while 2,6-dichloroindophenol, mitomycin C and mitoxantrone were added dissolved in distilled water. The reduction of DTNB was measured over 2 min, and the extinction coefficient of thionitrobenzoic acid at 412 nm was taken as 13,600 $M^{-1}cm^{-1}$ [37]. In some studies the preincubation and assay were conducted under anaerobic conditions after thoroughly gassing the medium with oxygen-free nitrogen, or in the presence of the oxygen radical scavengers SOD (10 μg/mL) or catalase (30 μg/mL). For Michaelis–Menten analysis of the kinetic data we used the Enzfitter computer program (Elsevier-Biosoft, Cambridge, U.K.). For studies of the covalent binding of quinone to the enzyme, 25 μg TR was incubated at room temperature with either $5 \times 10^{-4}$ M [$^{14}$C]diaziquone (13.6 mCi/mmol) or $10^{-5}$ M [$^{14}$C]doxorubicin (12.2 mCi/mmol) in 5 mL of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA and 3 mM NADPH, for 60 min. The mixture was then desalted by repetitive ultrafiltration and dilution with water using a Diaflo PM-10 filter membrane (Amicon, Danvers, MA). After lyophilization the entire sample was subjected to SDS–PAGE under non-reducing conditions [38]. The radioactivity on the gels was quantitated using a radioanalytical imaging system (Ambis Systems Co., San Diego, CA) for 10 hr. The efficiency and limits of detectability of the imaging system were determined using $^{14}$C-labeled radioactive standards.

Electron spin resonance (ESR) spectroscopic studies were conducted using a Bruker X-band ER200 spectrometer. The quinoids, at 2–4 mM, were incubated in 4 mL of 0.1 M potassium phosphate buffer, pH 7.0, 0.1 mM EDTA with 1 mM NADPH and 10 μg TR or 200 μg rat hepatic microsomal protein under aerobic or anaerobic conditions. The external field was swept repetitively every 3–4 min over 40–70 G, with a modulation amplitude of 0,8 to 1.6 G and a modulation frequency of 100 kHz.

Table 1. Inhibition of thioredoxin reductase by quinoids

| Compound | TR activity (% control) | |
|---|---|---|
| | 0 min | 60-min Preincubation |
| Quinones | | |
| Diaziquone | 63 | 6 |
| Doxorubicin | 56 | 4 |
| Menadione | 69 | 32 |
| Mitomycin C | 100 | 100 |
| Streptonigrin | 93 | 82 |
| Quinoneimines | | |
| Actinomycin D | 100 | 75 |
| 2,6-Dichloroindophenol | 27 | 4 |

Quinoids were added to 0.5 to 1 μg TR at a concentration of $10^{-5}$ M at the same time as 0.2 mM NADPH and 1 mM DTNB (0 min), or were preincubated with TR and NADPH for 60 min before DTNB was added to the incubation mixture (60-min preincubation). All incubations were conducted at room temperature. The rate of DTNB reduction was measured over 2 min. In the absence of quinoid, DTNB reduction was 39 μmol/min/mg at 0 min and 31 μmol/min/mg after a 60-min incubation of TR with NADPH. Values are the means of duplicate experiments and are expressed as a percent of the appropriate control without quinoid.

## RESULTS

*Inhibition of TR by quinoids.* Several quinoids were tested for their ability to inhibit the metabolism of DTNB by TR (Table 1). Diaziquone, doxorubicin,

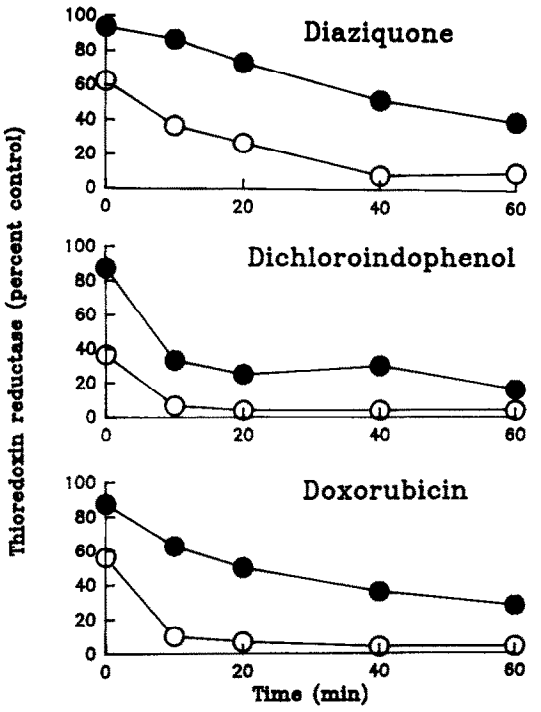


Fig. 1. Time course of the inhibition of thioredoxin reductase. Diaziquone, doxorubicin or 2,6-dichloroindophenol at $10^{-5}$ M (○) or $10^{-6}$ M (●) were incubated with 1 μg thioredoxin reductase and 0.2 mM NADPH for various periods up to 60 min before addition of 1 mM DTNB. In the absence of quinoid, DTNB reduction at 0 min was 38.6 ± 0.1 μmol/min/mg. Data are the means of duplicate experiments and are expressed as a percent of the appropriate non-quinoid exposed control value.

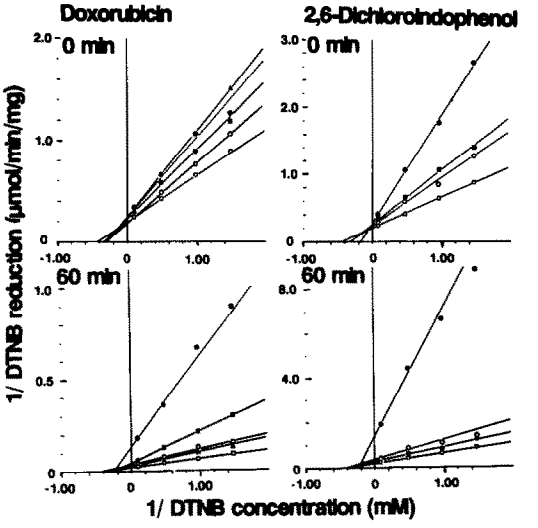


Fig. 2. Lineweaver–Burk plot of the kinetics of the inhibition of TR with DTNB as the substrate by doxorubicin and 2,6-dichloroindophenol. In the upper panels the quinoids were added at the same time as DTNB and 0.2 mM NADPH to 0.5 μg TR (0 min). In the lower panels the quinoids were added with NADPH to TR 60 min before the addition of DTNB. NADPH was not limiting for DTNB reduction. Studies were conducted at room temperature. Quinoid concentrations, in increasing order, each representing a plot on the graph are: doxorubicin 0 min, 0, $10^{-7}$ M, $10^{-6}$ M, $10^{-5}$ M, and $10^{-4}$ M; doxorubicin 60 min, 0, $10^{-8}$ M, $10^{-7}$ M, $10^{-6}$ M, and $10^{-5}$ M; 2,6-dichloroindophenol 0 min, $10^{-6}$ M, $2 \times 10^{-6}$ M and $10^{-5}$ M; 2,6-dichloroindophenol 60 min, 0, $10^{-8}$ M, $10^{-7}$ M, and $10^{-6}$ M. Data are the means of duplicate experiments. Continuous lines are Enzfitter computer program generated fits to the data.

2,6-dichloroindophenol and streptonigrin inhibited TR activity when added at the same time as DTNB. The inhibition became more pronounced after a 60-min preincubation of TR with NADPH and the quinoids. There was only a small loss of activity when TR was incubated for 60 min with NADPH alone (Table 1). In the absence of NADPH, there was no significant inhibition of TR over the 60-min incubation with the quinoids (results not shown). Diaziquone, doxorubicin and 2,6-dichloroindophenol were chosen for further study as they showed the greatest inhibition of TR activity.

There was a progressive time-dependent inhibition of TR by diaziquone, doxorubicin and 2,6-dichloroindophenol (Fig. 1) that occurred by first order processes. Adding an additional 0.2 mM NADPH at the end of the 60-min preincubation did not alter the inhibition of DTNB reduction by TR showing that NADPH was not limiting under these conditions (results not shown). Adding $10^{-6}$ M FAD to the incubation, which stabilizes some flavoenzymes [39], did not protect against inhibition of TR by the quinoids (results not shown). Extensive dialysis of TR that had been preincubated for 60 min with 0.2 mM NADPH and $5 \times 10^{-5}$ M diaziquone or $10^{-5}$ M doxorubicin, over 3 days against $9 \times 2$ L of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.2 mg/mL bovine serum albumin, $10^{-6}$ M FAD failed to reverse the inhibition of TR (results not shown).

Michaelis–Menten kinetic analysis of the inhibition of TR activity revealed that diaziquone without preincubation was a competitive inhibitor of DTNB reduction, with a $K_i$ of 7.5 μM, but became a noncompetitive inhibitor after a 60-min incubation with NADPH and the enzyme, with a $K_i$ of 0.5 μM. Doxorubicin without preincubation showed characteristics of both competitive and noncompetitive inhibition, with an approximate $K_i$ of 10 μM, but became a noncompetitive inhibitor after a 60-min incubation with NADPH and the enzyme, with a $K_i$ of 0.5 μM (Fig. 2). 2,6-Dichloroindophenol without preincubation was a competitive inhibitor, with a $K_i$ of 4.2 μM, but became a noncompetitive inhibitor after a 60-min incubation with NADPH and the enzyme, with a $K_i$ of 0.07 μM (Fig. 2).

*Free radical formation.* Incubation of diaziquone, doxorubicin, and 2,6-dichloroindophenol with TR and NADPH under anaerobic conditions gave ESR spectra characteristics of the diaziquone semiquinone free radical [40], the doxorubicin semiquinone free radical [41] and the 2,6-dichloroindophenol

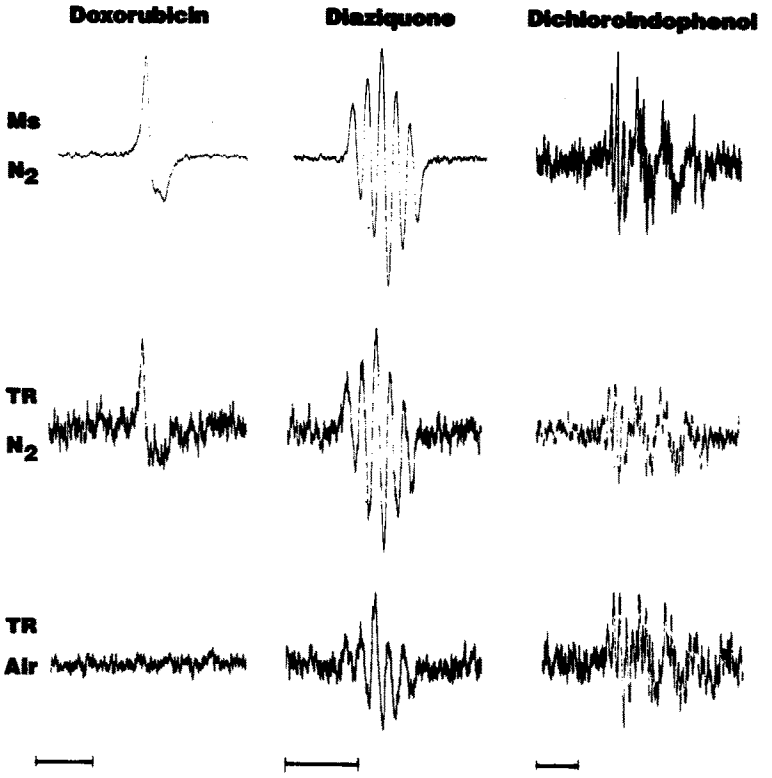


Fig. 3. ESR spectra of free radical formed by quinoids incubated with TR or hepatic microsomal fraction. Doxorubicin (2 mM), diaziquone (2 mM), or 2,6-dichloroindophenol (4 mM), in 0.1 M potassium phosphate buffer, pH 7.0, 0.1 mM EDTA were incubated with 10 μg/mL TR and 1 mM NADPH for 15 min under anaerobic ($N_2$) or aerobic (air) conditions, or with 200 μg/mL rat hepatic microsomal protein (Ms) and 1 mM NADPH for 5 min under anaerobic conditions. The instrument parameters were: sweep width 40–70 G; sweep time 3–4 min; time constant 0.6 sec; modulation frequency 100 kHz; modulation amplitude 0.8 to 1.6 G; gain, diaziquone $2 \times 10^5$; doxorubicin $8 \times 10^4$; 2,6-dichloroindophenol $2 \times 10^5$. Bars are 10 G. There was no signal with any quinoid without NADPH or enzyme.

semiquinoneimine free radical [42], respectively (Fig. 3). The doxorubicin semiquinone free radical signal was not seen under aerobic incubation conditions. However, a small diaziquone semiquinone free radical and a 2,6-dichloroindophenol semiquinoneimine free radical signal were seen under aerobic incubation conditions (Fig. 3). In these studies the formation of the semiquinoid free radicals by rat hepatic microsomal fraction and NADPH under anaerobic conditions were used as positive controls.

*Effects of $N_2$ and oxygen radical scavengers.* Preincubation of the quinoids with TR and NADPH, and the assay of TR activity in the absence of oxygen produced a significant but small (no more than 30%) protection against the inhibition of TR activity by the quinoids compared to preincubation and assays conducted in air (Table 2). The oxygen radical scavengers SOD and/or catalase had no significant effect upon inhibition of TR activity by the quinoids. There was also no effect of anaerobic conditions, or of SOD and/or catalase, on the inhibition of DTNB reduction by TR produced by the quinoids added at the same time as DTNB (results not shown).

*Covalent binding.* When TR was incubated with 3 mM NADPH and $5 \times 10^{-4}$ M [$^{14}$C]diaziquone for 60 min, the enzyme activity was found to be 97% inhibited. SDS–PAGE of the inhibited enzyme under nonreducing conditions, followed by radioanalytical scanning of the gels revealed 117 cpm bound (over background) to 25 μg TR protein. When NADPH was omitted from the incubation, the radioactivity bound was 111 cpm to 25 μg TR protein. When TR was incubated with 3 mM NADPH and $10^{-5}$ M [$^{14}$C]-doxorubicin for 60 min, the enzyme activity was found to be 91% inhibited. SDS–PAGE and radioanalytical scanning revealed no detectable radioactivity associated with the TR protein. The lower limit of detectability for radioactivity by the radioanalytical scanner under the conditions employed was found to be 23 cpm over background, and the efficiency was 10.4%. The theoretical amount of radioactivity bound to 25 μg TR that could be detected by the scanner, assuming a 1:1 complex between the enzyme and the radiolabeled quinoid, at the specific activity employed was calculated to be 1360 cpm for [$^{14}$C]diaziquone and 603 cpm for [$^{14}$C]doxorubicin. Thus, although

Table 2. Effects of nitrogen and oxygen radical scavengers on inhibition of TR by quinoids

| | TR activity (% control) | | | | | |
|---|---|---|---|---|---|---|
| | Diaziquone | | Doxorubicin | | 2,6-Dichloroindophenol | |
| Conditions | $10^{-5}$ M | $10^{-6}$ M | $10^{-5}$ M | $10^{-6}$ M | $10^{-5}$ M | $10^{-6}$ M |
| No addition (air) | 12 ± 2 | 48 ± 0 | 12 ± 1 | 23 ± 4 | 9 ± 1 | 12 ± 0 |
| $N_2$ | 27 ± 4* | 63 ± 4* | 29 ± 4* | 53 ± 6* | 15 ± 2 | 25 ± 2* |
| No addition (air) | 13 ± 1 | 52 ± 1 | 10 ± 1 | 23 ± 0 | 13 ± 2 | 11 ± 1 |
| SOD | 29 ± 2* | 42 ± 2* | 6 ± 1* | 15 ± 3 | 7 ± 1 | 10 ± 0 |
| Catalase | 37 ± 1* | 53 ± 1 | 11 ± 0 | 25 ± 1 | 8 ± 0 | 15 ± 2 |
| SOD + catalase | 32 ± 2* | 46 ± 2 | 8 ± 0* | 22 ± 1 | 8 ± 1 | 13 ± 0 |

The quinoids at $10^{-5}$ or $10^{-6}$ M were preincubated for 60 min with 0.2 mM NADPH and 1 μg TR in air, or under $N_2$ before measuring TR activity. In the absence of quinoid, DTNB reduction after 60 min in air was 46 ± 1 μmol/min/mg and after 60 min in $N_2$ 36 ± 4 μmol/min/mg ($P > 0.05$). In other studies, superoxide dismutase (SOD, 10 μg/mL), catalase (30 μg/mL), or a combination of SOD and catalase were present during the preincubation in air. In these studies DTNB reduction in the absence of quinoids and the scavengers was 32 ± 1 μmol/min/mg. Values are the means of triplicate determinations ± SEM and are expressed as a percent of the appropriate control value without quinoid.

* $P < 0.05$ compared to appropriate control value.

theoretically possible, we were unable to detect specific NADPH-dependent binding of doxorubicin and diaziquone to TR in our studies.

## DISCUSSION

Antitumor quinones can be reduced chemically or enzymatically to give reactive intermediates that form covalent adducts with tissue macromolecules including DNA [3, 16, 22], or that react with molecular oxygen to give reactive oxygen species [18, 19, 23]. A number of flavoenzymes catalyze the reduction of antitumor quinones [8, 18]. If the quinoid reactive intermediates achieve appreciable concentrations in the vicinity of the enzyme, the flavoenzyme itself could be inhibited during metabolism of the antitumor quinone. Furthermore, it is possible to envision that, if the flavoenzyme is important for cell proliferation, the inhibition may contribute to the growth inhibitory activity of the antitumor quinones. There is some evidence for inhibition of flavoenzymes by antitumor quinones. Doxorubicin has been reported to inhibit beef heart metmyoglobin reductase [43]. Ametrantone, an antitumor bis(substituted)aminoalkylaminoanthraquinone, inhibits NADPH–cytochrome P450 reductase [44]. However, inhibition in these cases was suggested to be due to displacement of flavin from the apoenzyme by the quinone [44, 45].

We chose to study the flavoenzyme TR because of its important role in a number of aspects of cell function [28, 29, 46]. Mammalian TR has a wide substrate specificity and has been reported previously to metabolize at least one quinone, menadione [35]. We found that a number of antitumor quinones and quinoneimines caused the NADPH and time-dependent inhibition of rat liver TR. The most active compounds were the quinones diaziquone and doxorubicin, which have marked antitumor activity in animal models and against some human cancers [1], and the quinoneimine 2,6-dichloroindophenol, which has weak antitumor activity in animal models [47]. The antitumor quinones mitomycin C and mitoxantrone were not inhibitors of TR. This may be because these quinones were poor substrates for metabolism by TR. Mitomycin C is not a substrate for reduction by some flavoenzymes at physiological pH [39], and mitoxantrone is a poor substrate for reduction by flavoenzymes because of its low oxidation–reduction potential [8]. It should be noted that a loss of TR activity has been reported previously upon incubation of the enzyme with NADPH alone [48]. However, EDTA gave full protection against this loss of TR activity [48] and EDTA was routinely present in all our incubations. EDTA, however, did not appear to protect against the quinoid-dependent loss of TR activity.

The inhibition of TR by quinoids has characteristics of mechanism-based or suicide-substrate inhibition [49, 50]. In mechanism-based inhibition the substrate is metabolized by the enzyme to give a reactive intermediate which irreversibly inactivates the enzyme. Sjoerdsma [51] has proposed a number of criteria that identify mechanism-based inhibition of enzyme activity. These are: that the loss of enzyme activity should be dependent upon metabolism and that it is time dependent, first order and irreversible. We found that the inhibition of TR by the antitumor quinoids was time dependent, first order and was not reversed by extensive dialysis of the inhibited enzyme. An additional criterion given by Sjoerdsma [51] for mechanism-based enzyme inhibition is protection against the inactivation of the enzyme by the natural substrate in order to demonstrate involvement of the active site of the enzyme. We were unable to test this possibility because of the unavailability of rat thioredoxin.

The inhibition of TR by the quinoids involved metabolism as shown by the requirement for

NADPH, and the kinetics of the inhibition that changed from initially competitive or partially competitive, to noncompetitive after the 60-min incubation of the enzyme with quinoid and NADPH. Oxygen reactive species formed by redox cycling of the semiquinoid free radical do not appear to be a major factor responsible for inhibition of TR. First, TR inhibition by the quinoids was decreased less than 30% in the absence of molecular oxygen. Second, 2,6-dichloroindophenol, which we found to be a potent inhibitor of TR, is reported not to undergo redox cycling when reduced [52]. Third, the oxygen radical scavengers SOD and catalase [53] did not uniformly protect against inhibition of TR by the quinoids. However, it is possible that SOD and catalase, being relatively large molecules, might not have had access to a site where oxygen radicals were formed. It should be noted that the substrate DTNB, doxorubicin, and diaziquone were added dissolved in dimethyl sulfoxide which is a known hydroxyl radical scavenger [54]. It is not known whether, under the conditions of the assay, all hydroxyl radicals could be scavenged. For mechanism-based inhibition of an enzyme to occur inactivation of the enzyme by the metabolic intermediate must occur prior to the release of the intermediate from the active site [25]. Although the activated species can be released by the enzyme, this will reduce the efficiency of inactivation. The quinoids were found to be metabolized by TR to semiquinoid radical intermediates. These intermediates are well known to give rise to reactive intermediates [1] that could bind to and inactivate the enzyme.

Azelaic acid and other saturated dicarboxylic acids have been reported to be reversible, competitive inhibitors of TR [55]. Interestingly, long incubation times were necessary to obtain the maximum inhibition of TR by azelaic acid. Nitrosourea anticancer drugs have also been reported to inhibit TR, probably due to alkylation of the thiolate active site of the enzyme [56]. 13-*cis*-Retinoic acid is a potent inhibitor of TR [57]. Covalent binding of radiolabeled 13-*cis*-retinoic acid to the enzyme was demonstrated and a mechanism-based inhibition was suggested. In our studies we were unable to demonstrate significant metabolism-dependent binding of radiolabeled diaziquone or doxorubicin to the inhibited TR protein. For mechanism-based enzyme inhibition a 1:1 stoichiometry of inactivator to the active site would be expected [25]. We observed limited covalent binding of radiolabeled diaziquone to TR that was not dependent on NADPH. In any case, this binding was too small to account for inhibition of the enzyme by a 1:1 complex with the quinone. Diaziquone is a direct alkylating agent at neutral pH due to proton catalyzed activation of its aziridine groups [58, 59] and was probably binding to TR at sites other than the enzyme active site. We cannot rule out the possibility that the quinoids formed a weak covalent bond with TR that was broken during SDS–PAGE. Another possibility is that the quinoids bind to, or in some other way inactivate the FAD prosthetic group of TR. Antitumor quinones have been suggested to bind to flavin prosthetic groups of flavoenzymes [60]. This possibility appears to be ruled out in the present study by the failure of FAD to protect TR against inhibition by the quinoids, or to restore TR activity in enzyme that had been inhibited and the quinoid then removed by extensive dialysis. This assumes, however, that a putative FAD–quinoid complex is free to leave the native enzyme and can be replaced by exogenous FAD, which may not be the case. On the other hand, the FAD–quinone complex would be expected to readily dissociate from the denatured enzyme, which might explain our inability to detect covalent binding of the quinones to inhibited TR by SDS–PAGE.

In summary, the results of the study show that some antitumor quinones and quinoneimines have characteristics of mechanism-based inhibitors of TR. The inhibition was irreversible, first order and dependent upon the reduction of the quinoid in the presence of NADPH. The formation of oxygen radicals by redox cycling of the quinoids did not appear to play a major role in the inhibition of TR. TR reduced the quinoids to ESR detectable semiquinoid free radicals in the presence of NADPH. It was not possible to establish whether inhibition of the enzyme was due to the formation of a covalent complex between the quinoid and the TR apoprotein or the FAD prosthetic group. TR is an important enzyme for a number of cellular functions, and further studies will be necessary to determine the biological significance, if any, of TR inhibition by antitumor quinone in intact cells.

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