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INHIBITION OF CELLULAR THIOREDOXIN REDUCTASE BY DIAZIQUONE AND DOXORUBICIN

RELATIONSHIP TO THE INHIBITION OF CELL PROLIFERATION AND DECREASED RIBONUCLEOTIDE REDUCTASE ACTIVITY

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Abstract—The flavoenzyme thioredoxin reductase (TR) is an important enzyme for many aspects of cellular function. The antitumor quinones diaziquone and doxorubicin have been shown to produce a time- and concentration-dependent inhibition of TR when incubated for up to 24 hr with intact A204 human rhabdomyosarcoma cells. There was a positive correlation between the inhibition of TR and the inhibition of cell colony formation measured 7 days later for diaziquone ($r = 0.84$, $P < 0.01$), and for doxorubicin ($r = 0.87$, $P < 0.01$). 2,6-Dichloroindophenol, which in previous studies was shown to be a good inhibitor of TR *in vitro*, was a poor inhibitor of TR in intact A204 cells and there was no significant correlation with inhibition of colony formation. The activity of ribonucleotide reductase, which catalyzes the first unique step of DNA synthesis and which obtains its reducing equivalents from TR through thioredoxin, was decreased in diaziquone- and doxorubicin treated A204 cells. We suggest that the inhibition of TR by some antitumor quinones leading to a decreased activity of TR and, consequently, a decreased activity of thioredoxin-dependent enzymes including ribonucleotide reductase may contribute to the growth inhibitory activity of these quinones.

Thioredoxin reductase (EC 1.6.4.5; TR†) is a flavoenzyme found in nearly all living cells [1]. The natural substrate of TR is the redox protein thioredoxin which is kept in its dithiolreduced form by TR in the presence of NADPH. Together TR, NADPH and thioredoxin comprise the thioredoxin system which provides an important source of reducing equivalents in mammalian cells for scavenging free radicals [2], as an endogenous activating factor of the cytosolic glucocorticoid receptor [3] and as a source of electrons for a number of enzymes including protein disulfide isomerase [4], vitamin K epoxide reductase [5], and ribonucleotide reductase [1]. Recently thioredoxin-like domains have been found in phosphoinositide phospholipase C-α [6], in adult T-cell leukemia-derived factor [7] and in the gonadotropic hormones follitropin and luteotropin [8], suggesting that the thioredoxin system may be important for the action of these proteins.

We have shown recently that some antitumor quinoids, including the quinones diaziquone and doxorubicin and the quinoneimine 2,6-dichloroindophenol, are mechanism-based inhibitors of TR *in vitro* [9]. In the presence of NADPH these quinoids lead to a time-dependent, irreversible inhibition of purified rat liver TR. However, it was not established by these studies whether inhibition of TR by the quinoids could occur in intact cells, and the consequences for cellular function are unknown. Given the apparent importance of the thioredoxin system for many different aspects of cellular function, including processes necessary for cell proliferation, it might be anticipated that inhibition of TR could contribute to the growth inhibitory activity of the antitumor quinoids. We now report the inhibition of TR in intact cells by diaziquone, doxorubicin and 2,6-dichloroindophenol and demonstrate a casual relationship between the inhibition of TR and the inhibition of cell growth by the antitumor quinones, but not by 2,6-dichloroindophenol which was a relatively weak inhibitor of TR in intact cells. The activity of ribonucleotide reductase, which as noted above depends on the thioredoxin system for its supply of reducing equivalents, was inhibited in the intact cells by doxorubicin and diaziquone.

MATERIALS AND METHODS

Cells and chemicals. A204 human rhabdomyosarcoma cells were obtained from the American Tissue Type Culture Collection (Rockville, MD) and maintained in bulk culture in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were harvested with 0.05% trypsin, 0.02% EDTA and were used between passages 25 and 37.

Doxorubicin, 2,6-dichloroindophenol, hydroxyurea, bovine insulin, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), NADPH and HEPES (*N*-(2-hyd-

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† Abbreviations: TR, thioredoxin reductase; FBS, fetal bovine serum; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; DMEM, Dulbecco's Modified Eagle's Medium; PBS, Dulbecco's phosphate-buffered saline; and RR, ribonucleotide reductase.

roxyethyl) piperazine-*N'*-2-ethanesulfonic acid) were obtained from the Sigma Chemical Co. (St. Louis, MO). Recombinant *Escherichia coli* reduced thioredoxin was obtained from Calbiochem (San Diego, CA). Diaziquone was provided by the Division of Drug Treatment, National Cancer Institute (Bethesda, MD). [U-^{14}C]Cytidine (400 mCi/mmol) was obtained from CEN Saclay (Yvette, France).

Procedures. A204 cells were exposed to the quinoids for 1, 4, and 24 hr. For the 1- and 4-hr exposures, suspensions of A204 cells, 4×10^6 cells/10 ml DMEM containing 10% FBS, were incubated with the quinoids with gentle shaking under humidified 5% CO_2:95% air at 37°. The cells were then washed three times with Dulbecco's phosphate-buffered saline (PBS) before assaying (see following). For the 24-hr quinoid exposure cells were first seeded at 2.5×10^4 cells/cm^2 in 150 cm^2 culture flasks with 20 ml DMEM containing 10% FBS under humidified 5% CO_2:95% air at 37° and allowed to attach to the flask for 24 hr. The medium was then replaced with medium containing the quinoids and the cells were exposed for 24 hr. The cells were washed three times with PBS, harvested with PBS containing 0.1% EDTA, and washed again with PBS. Cells from both the short- and long-term incubations were suspended in DMEM, and an aliquot of the cells was taken for measurement of cell colony formation and the remainder for assay of TR. Colony formation was measured using the soft agarose colony forming assay as previously described [10]. Colonies were counted after 7 days of growth using a Bausch and Lomb FAS-II image analysis system and only colonies larger than 60 μm in diameter were counted. Colony formation was expressed relative to that of nontreated controls and all quinoid concentrations were studied with triplicate cultures.

For the assay of TR, quinoid-exposed and non-treated cells were suspended at 2×10^6 cells/1 mL of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA and sonicated for three bursts of 10 sec each at 4°. TR was measured by a modification of the insulin reduction assay using DTNB as the final substrate as described by Holmgren [11]. The use of insulin as the primary substrate for reduction by the thioredoxin system was necessary to prevent the reduction of DTNB by other reductases and endogenous thiols in the cell homogenate. For the assay 10–100 μL cell homogenate was mixed with 240 μL of 83 mM HEPES buffer, pH 7.6, 5 mM EDTA, 0.9 mM NADPH, 2.08 mg/ml insulin and 10 μM reduced *E. coli* thioredoxin. After a 15-min incubation at 37°, 1 mL of 6 M guanidine hydrochloride in 50 mM Tris buffer, pH 8.0 containing 10 mM DTNB was added and the mixture shaken thoroughly for 1 min. The formation of thionitrobenzoic acid from DTNB was measured spectrophotometrically at 412 mm using an extinction coefficient for thionitrobenzoic acid of 13,600 $M^{-1}\,cm^{-1}$ [12]. The background reduction of DTNB by endogenous thiols was measured by omitting NADPH, insulin, and thioredoxin from the incubation mixture and this value was subtracted to obtain the value for the specific reduction of DTNB by TR-reduced insulin. Preliminary studies showed that the assay was linear using up to 100 μL of A204 cell homogenate.

The ribonucleotide reductase activity of intact A204 cells was measured by following the conversion of [^{14}C]cytidine to [^{14}C]deoxycytidine nucleotides, and the incorporation of [^{14}C]cytidine into RNA and of [^{14}C]deoxycytidine into DNA, as described by Cory *et al.* [13]. Briefly, cultures of A204 cells were exposed to quinones for 24 hr as described previously. For the last 30 min of the incubation [^{14}C]cytidine, 1.5 μCi, 3.75 nmol/150-cm^2 flask, was present. After washing the cells three times with PBS and harvesting with PBS containing 0.1% EDTA, the cell pellet was extracted three times with 6% perchloric acid. The acid supernatant was neutralized using 30% KOH and, after removing the precipitated $KClO_4$, the supernatant was incubated for 4 hr at 37° with 3 mg Crotalus adamanteous snake venom in Tris buffer, pH 8.8, containing 6 mM 2′-deoxycytidine 5′-monophosphate and 2 mM $MgCl_2$. The reaction was terminated by boiling for 4 min and [^{14}C]-deoxycytidine was separated from [^{14}C]cytidine by binding to a 0.5 × 10 cm Dowex-1 -borate column and eluting with 3 ml H_2O. RNA in the acid-insoluble pellet was hydrolyzed with 200 μL 0.5 M NaOH at 65° for 1 hr. DNA in the supernatant was precipitated after mixing with 50 μg salmon sperm DNA, by neutralizing with 6 N HCl, mixing with 1 ml of 10% trichloroacetic acid at 4° and collecting the precipitate on a Whatman GF/C microfiber filter. Ribonucleotide reductase activity was measured as the ratio of the radioactivity in [^{14}C]deoxycytidine nucleotides (free and DNA) to the radioactivity in [^{14}C]cytidine nucleosides (free and RNA).

RESULTS

Inhibition of A204 cell TR by quinoids. A204 human rhabdomyosarcoma cells were exposed to various concentrations of the quinoids for up to 24 hr. Diaziquone, doxorubicin and 2,6-dichloro-indophenol all inhibited cellular TR with greater inhibition being seen with longer times of exposure (Fig. 1). With diaziquone and 2,6-dichloroindophenol the maximum inhibition of TR occurred after a 4-hr exposure, but with doxorubicin there was a progressive inhibition of TR over 24 hr of exposure. The inhibition of TR was found to be concentration dependent for all three quinoids and the IC_{50} values for maximum inhibition of TR were: for diaziquone, 2.5×10^{-5} M; for doxorubicin, 4×10^{-6} M; and for 2,6-dichloroindophenol 10^{-4} M.

Inhibition of colony formation. The ability of the quinoids to inhibit A204 cell colony formation was studied using aliquots of cells removed from the same incubations used to study inhibition of TR so that inhibition of TR could be correlated with the inhibition of cell colony formation measured 7 days later (Fig. 2). Significant positive correlations were obtained for diaziquone ($r = 0.84$, $P = 0.004$), and doxorubicin ($r = 0.87$, $P = 0.002$). With both quinoids there was approximately 50% inhibition of colony formation before inhibition of TR became apparent. Inhibition of colony formation was also seen with 2,6-dichloroindophenol at concentrations above 10^{-4} M, but the correlation between the

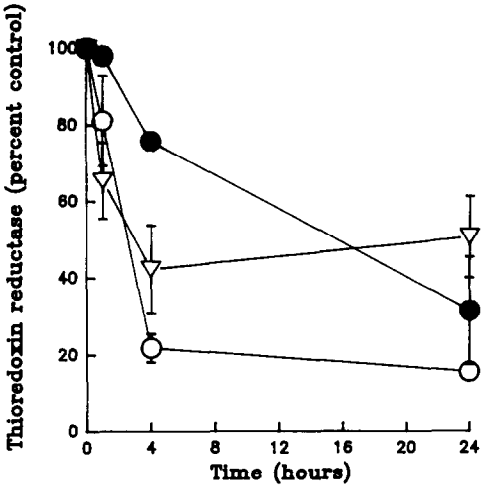

Fig. 1. Inhibition of TR in A204 cells exposed to antitumor quinoids. Cells in culture were exposed to 5×10^{-5} M diaziquone (○), 2×10^{-6} M doxorubicin (●) and 10^{-4} M 2,6-dichloroindophenol (▽) for 1, 4, or 24 hr. After washing to remove quinoid, the cells were lysed and TR activity was measured. Values are expressed as a percentage of the nontreated TR value which was 1.7 ± 0.1 nmol DTNB reduced/min/mg protein. Each point is the mean of three separate experiments and bars are SEM.

inhibition of TR and the inhibition of colony formation was not signficant ($r = 0.42$, $P = 0.26$). (data not shown).

Inhibition of ribonucleotide reductase. A204 cells were exposed to various concentrations of diaziquone or doxorubicin for 24 hr and ribonucleotide reductase activity was measured in the intact cells by following the conversion of [^{14}C]cytidine to [^{14}C]deoxycytidine, and their incorporation into RNA and DNA, respectively. Both diaziquone and doxorubicin caused a concentration-dependent inhibition of ribonucleotide reductase activity (Fig. 3). Exposure of A204 cells to 10^{-4} M hydroxyurea, a known inhibitor of ribonucleotide reductase, for 2 hr as a positive control [14] gave 82% inhibition of ribonucleotide reductase activity.

DISCUSSION

Antitumor quinones such as diaziquone and doxorubicin have many actions in the cell. These include intercalation and covalent binding to DNA [15–17], inhibition of nuclear DNA topoisomerase II [18], redox cycling with the formation of reactive oxygen species [19, 20] and membrane-altering properties [21, 22]. It has been difficult to demonstrate a direct relationship between the inhibition of cell proliferation and a single mechanism of action of the antitumor quinones [23–26]. The anthracyclines exhibit greater toxicity to cells than can be accounted for by DNA cleavage alone [27]. The present studies show that diaziquone and doxorubicin can also inhibit TR in intact cells. There were significant, positive correlations between the inhibition of TR in A204 cells by the quinones, and the inhibition of colony formation in the same cells measured seven days later. About half of the growth inhibition caused by the antitumor quinones occurred before inhibition of TR became apparent. This could represent the different conditions under which the

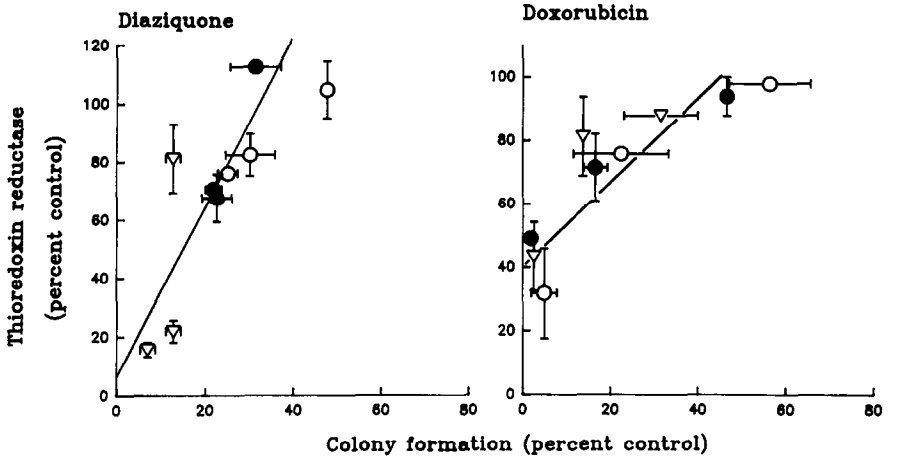

Fig. 2. Correlation between the inhibition of A204 cell TR by quinoids and the inhibition of colony formation in the same cells. Cells were exposed to, left panel, diaziquone at 5×10^{-6} M (○), 10^{-5} M (●) and 5×10^{-5} M (▽); and, right panel, to doxorubicin at 2×10^{-6} M (○), 4×10^{-6} M (●) and 6×10^{-6} M (▽) for 1, 4, or 24 hr. After washing to remove the quinoid, some of the cells were taken for measurement of TR and some of the cells were plated in soft agarose for measurement of colony formation 7 days later. Each point is the mean of three separate experiments and bars are SEM. Values are expressed as a percentage of nontreated controls. The value for TR in nontreated cells was 1.7 ± 0.1 nmol DTNB reduced/min/mg protein. The continuous lines are computer generated regression fits to the data.

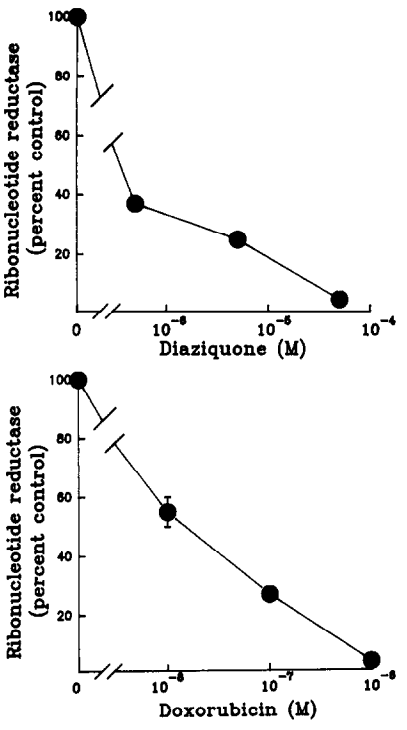

Fig. 3. Inhibition of ribonucleotide reductase activity in A204 cells by diaziquone and doxorubicin. Cells were exposed to diaziquone or doxorubicin at various concentrations for 24 hr and cellular ribonucleotide reductase activity was measured by the conversion of [^{14}C]cytidine to [^{14}C]deoxycytidine nucleotides (free and DNA) expressed as a ratio to the radioactivity in [^{14}C]cytidine nucleosides (free and RNA). The mean ratio in nontreated cells was (± SEM) $7.8 \pm 1.7 \times 10^{-3}$. Values are shown expressed as a percentage of the ratio in nontreated cells. Each point is the mean of three experiments and bars are SEM.

two assays were conducted (TR inhibition was measured acutely, cell colony formation was measured after 7 days), but it is more likely that several mechanisms, including possibly inhibition of TR, contribute to the inhibition of cell proliferation by the antitumor quinones.

We have reported previously that both diaziquone and doxorubicin are mechanism based inhibitors of purified rat liver TR producing NADPH and time-dependent, irreversible inhibition of the enzyme [9]. While we do not know whether the same mechanism of TR inhibition by the quinones is operative in intact A204 cells, we did observe that the TR inhibition was time dependent, reaching a maximum after 4 hr with diaziquone and increasing to 24 hr with doxorubicin. However, the possibility that this time dependence reflects the accumulation of the quinones by the cells, particularly for doxorubicin which is accumulated by many cell types [28], cannot be ruled out.

We have reported previously that 2,6-dichloroindophenol, a quinoneimine with weak antitumor activity [29], is a potent inhibitor of TR *in vitro* [9]. The K_i for inhibition of TR by 2,6-dichloroindophenol after a 60-min incubation with NADPH and the enzyme was 0.07 μM, compared to 0.5 μM for both diaziquone and doxorubicin [9]. However, 2,6-dichloroindophenol was found to be a poor inhibitor of TR in intact A204 cells with an IC_{50} of 10^{-4} M, compared to 2.5×10^{-5} M for diaziquone and 4×10^{-6} M for doxorubicin. 2,6-Dichloroindophenol was not growth inhibitory for A204 cells at concentrations below 10^{-4} M, and the inhibition of TR showed no significant correlation with the growth inhibition by 2,6-dichloroindophenol. The reason 2,6-dichloroindophenol is a poor inhibitor of TR in intact cells may be that it is metabolized. Normal and transformed cells can rapidly metabolize 2,6-dichloroindophenol [30]. Enzymes that metabolize 2,6-dichloroindophenol include methemoglobin reductase [31] and NAD(P)H:(quinone-acceptor)-oxidoreductase (QAO) [32]. 2,6-Dichloroindophenol is, in fact, one of the best known electron acceptors for QAO [32] and A204 cells contain relatively high levels of QAO [33]. Although diaziquone is reduced by QAO [34], it is a relatively poor substrate compared to 2,6-dichloroindophenol, while doxorubicin is not a substrate for QAO at physiological pH ([35], Schlager J and Powis G, unpublished observations).

To determine whether inhibition of TR in intact cells could lead to the inhibition of TR-dependent cellular processes, we studied the activity of ribonucleotide reductase in A204 cells. Reduced thioredoxin provides a source of reducing equivalents for ribonucleotide reductase [36]. Although in bacteria an alternate system with glutaredoxin as the final electron acceptor can provide reducing equivalents for ribonucleotide reductase [37], it appears that in mammalian cells thioredoxin cannot be substituted by glutaredoxin as a source of reducing equivalents for ribonucleotide reductase [38, 39]. Ribonucleotide reductase catalyzes the first unique and rate-limiting step in the synthesis of DNA [37] and the direct inhibition of ribonucleotide reductase by hydroxyurea leads to inhibition of cell proliferation [14, 40]. Thus, the inhibition of ribonucleotide reductase caused by an inhibition of TR might provide a link to the inhibition of cell proliferation by the antitumor quinones. Although neither diaziquone nor doxorubicin inhibits ribonucleotide reductase directly [41, 42], we found that in intact A204 cells treated with diaziquone or doxorubicin there was a concentration-dependent inhibition of ribonucleotide reductase activity. Presumably, inhibition of TR by the quinones led to decreased levels of reduced thioredoxin that acts as a source of reducing equivalents for ribonucleotide reductase. However, reduced thioredoxin levels were not measured directly in this study. There are reports of other *in vitro* inhibitors of TR including azelaic acid [43], 13-*cis*-retinoic acid [44] and nitrosourea anticancer drugs [45]. It has been suggested that inhibition of TR and the consequent inhibition of ribonucleotide reductase activity in intact cells could explain the inhibition of DNA synthesis by these agents. However, the direct demonstration of inhibition of cellular TR and, more important, a decrease in cellular ribonucleotide reductase activity were not made in these studies. We have now shown

using antitumor quinones as mechanism-based inhibitors of TR that such a mechanism may occur in intact cells.

In summary, we have shown that the antitumor quinones diaziquone and doxorubicin are concentration- and time-dependent inhibitors of TR in A204 cells. The inhibition of TR correlated with a decrease in colony formation of the quinone-treated cells, although other mechanisms of inhibition of colony formation were also operative. 2,6-Dichloroindophenol, which is a good inhibitor of TR *in vitro*, was a poor inhibitor of TR in A204 cells and only weakly inhibited colony formation. The activity of the thioredoxin-dependent enzyme ribonucleotide reductase was decreased in diaziquone- and doxorubicin-treated cells. The observation of the inhibition of TR by some antitumor quinones provides a new mechanism that may contribute to their growth inhibitory activity.

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