The antitumor anthracyclines doxorubicin and daunorubicin do not inhibit cell growth through the formation of iron-mediated reactive oxygen species

Xing Wu[a] and Brian B. Hasinoff[a]

The use of the anthracycline anticancer drugs doxorubicin and daunorubicin is limited by what is thought to be an iron-based oxygen radical-derived dose-dependent cardiotoxicity. The anthracyclines are also DNA topoisomerase (Topo) II poisons. It is not known if iron-mediated formation of reactive oxygen species (ROS) by the anthracyclines or their Topo II inhibitory effects are responsible for their cell growth-inhibitory effects. Experiments to test these two alternatives were carried out using a CHO-derived cell line (DZR) that was highly resistant to dexrazoxane through a Thr48Ile mutation in Topo IIα. The clinically used cardioprotective agent dexrazoxane likely exerts its cardioprotective effects through the chelating ability of its hydrolysis product ADR-925, an analog of EDTA. Dexrazoxane is also a cell growth inhibitor that acts through its ability to inhibit the catalytic activity of Topo II. Thus, the DZR cell line allowed us to examine the cell growth-inhibitory effects of doxorubicin and daunorubicin in the presence of dexrazoxane without the confounding effect of dexrazoxane inhibiting cell growth. The growth-inhibitory effects of neither doxorubicin nor daunorubicin were affected by pretreating DZR cells with dexrazoxane. In contrast, under similar conditions, dexrazoxane strongly protected rat cardiac myocytes from doxorubicin-induced lactate dehydrogenase release. In conclusion, the anthracyclines do not inhibit the growth of DZR cells through the generation of iron-mediated formation of ROS. *Anti-Cancer Drugs* **16:93–99 © 2005 Lippincott Williams & Wilkins.**

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Introduction

It is still commonly held that the cell growth-inhibitory effects of the anthracyclines against tumor cells is due to their ability to generate damaging free radical species. This is in spite of the fact that doxorubicin [1] and daunorubicin [1,2], like etoposide, mitoxantrone and amsacrine, have been shown to stabilize a covalent topoisomerase (Topo) II–DNA intermediate (the cleavable complex) [3], and thus act as a cellular poisons. The controversy surrounding the mechanism of the action of the anthracyclines has been addressed in recent reviews [4–7] with the stress on studies conducted with pharmacological concentrations of anthracycline. As has been noted in these reviews, multiple mechanisms may be involved in both their antitumor and cardiotoxic effects [4–7].

DNA Topo II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix [3]. The bisdioxopiperazines such as dexrazoxane (Fig. 1; ICRF-187, Zinecard) are strong catalytic inhibitors of Topo II [8,9]. Dexrazoxane acts by inhibiting cell division and induces endopolyploidy [10]. Unlike the Topo II poisons, the bisdioxopiperazines act without promoting significant cleavable complex formation [8]. We [11–13] and others [14] have shown that dexrazoxane is able to antagonize the growth-inhibitory effects and cleavable complex formation induced by anthracyclines and other Topo II poisons.

Doxorubicin can also be reductively activated [7,15–17] by several reductases such as NADPH cytochrome P450 reductase, xanthine oxidase and NADH dehydrogenase to its semiquinone free radical form. This semiquinone can react rapidly with oxygen to produce superoxide, hydrogen peroxide and ultimately the extremely damaging hydroxyl radical by reaction with the iron(II)–doxorubicin complex [18,19]. Thus, oxidative damage to sensitive cell components may also be a potential mechanism by which tumor cell growth is inhibited.

The clinical use of the antitumor anthracyclines such as doxorubicin is limited by a unique cumulative

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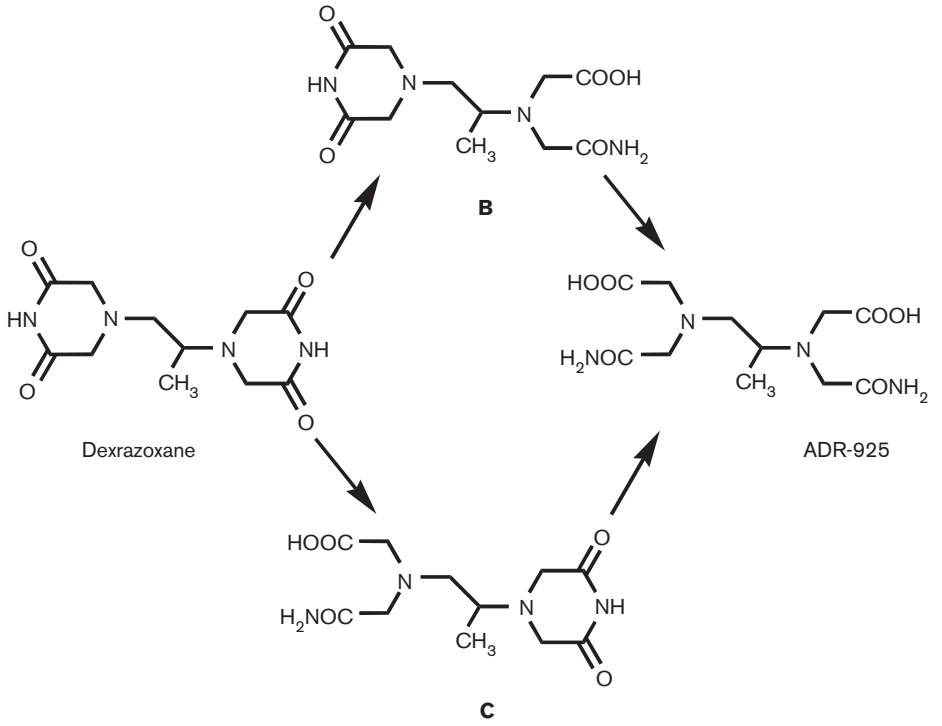
Fig. 1



Structure of dexrazoxane, its one-ring open hydrolysis products **B** and **C**, and its metal ion chelating EDTA-type hydrolysis product ADR-925.

dose-limiting cardiotoxicity [20,21]. There is now a considerable body of evidence to suggest that the cardiotoxicity may be due to iron-based oxygen free radical-induced oxidative stress [6,20,22] on the relatively unprotected heart muscle. Using electron paramagnetic resonance spectroscopy we previously showed that cardiac myocytes could reduce doxorubicin to form the doxorubicin semiquinone free radical species under hypoxic conditions [17]. Dexrazoxane is clinically used to reduce the cardiotoxicity of the anticancer drug doxorubicin [23,24]. Dexrazoxane likely acts by diffusing into the cell and hydrolyzing [23,25–27] to its rings-opened, iron-binding form ADR-925 (Fig. 1), which has a structure similar to EDTA. ADR-925 may then either remove iron from the iron–doxorubicin complex [28], or bind free or loosely bound iron, thus preventing site-specific iron-based oxygen radical damage. Thus, dexrazoxane, which is easily permeable to cells [29], can be considered a neutral pro-drug analog of EDTA.

We have characterized a CHO-derived cell line (DZR) with 1500-fold acquired resistance to dexrazoxane [13,30]. The DZR cell line has a Thr48Ile mutation in Topo IIα [13,30] that is located in the N-terminal ATP-binding region of Topo IIα close to the recently determined dexrazoxane-binding site [31,32] and likely sterically interferes with its binding. Thus, the DZR cell line, which can be grown in the presence of normally cytotoxic concentrations of dexrazoxane, allowed us to examine the cell growth-inhibitory effects of doxorubicin and daunorubicin under conditions that dexrazoxane is known to prevent iron-mediated oxygen radical damage to cardiac myocytes [17,33,34]. Thus, the use of the DZR cell line allowed us to determine whether any of the growth-inhibitory effects of the anthracyclines are due to iron-mediated formation of reactive oxygen species.

Materials and methods

Materials

Doxorubicin hydrochloride and dexrazoxane hydrochloride were gifts from Pharmacia & Upjohn (Columbus, OH). Daunorubicin was a gift from Rhône-Poulenc Pharma (Montreal, Canada). Trypsin, collagenase and DNase were from Worthington (Freehold, NJ). Unless specified, other reagents were obtained from Sigma (Oakville, Canada).

Myocyte isolation and culture

Ventricular myocytes were isolated from 1- to 3-day-old Sprague-Dawley rats as described [17,34,35]. Briefly, minced ventricles were serially digested with collagenase and trypsin in PBS/1% (w/v) glucose at 37°C in the presence of DNase and pre-plated in large Petri dishes to

remove fibroblasts. The preparation, which was typically greater than 90% viable by Trypan blue exclusion, yielded an almost confluent layer of uniformly beating heart myocytes by day 2. For the lactate dehydrogenase (LDH) release experiments the myocyte-rich supernatant was plated on day 0 in 24-well plastic culture dishes (5×10^5 myocytes/well, 750 µl/well). On day 2 and 3 the medium was replaced with 750 µl of fresh medium containing 10% (v/v) FBS. In order to lower the background LDH levels, on day 4, 24 h before the drug treatments, the medium was replaced with medium containing 2% (v/v) FBS and again on day 5 just before the addition of drugs. The animal protocol was approved by the University of Manitoba Animal Care Committee.

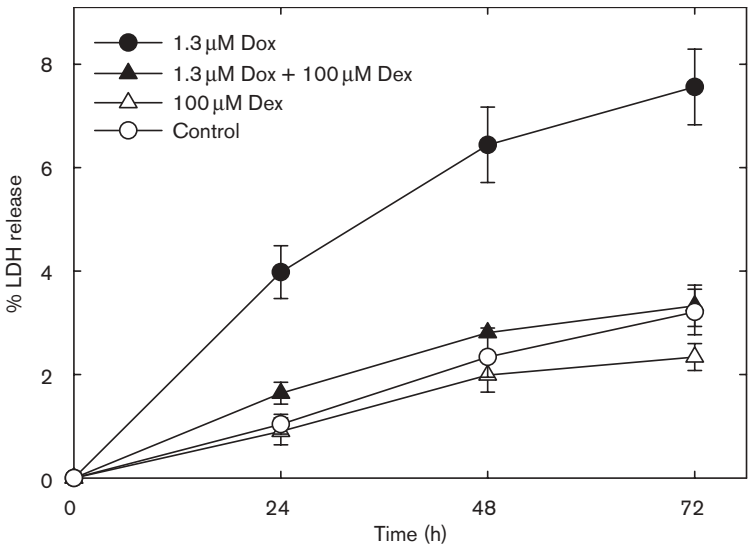
LDH determination

LDH released into the myocyte growth medium was determined as previously described [17,34,35]. Starting on day 6 after plating, samples (80 µl) of the myocyte supernatant were collected every 24 h for 3 days after treatment. The samples were frozen at −80°C and analyzed within 1 week. After the last supernatant sample was taken, the myocytes were lysed with 250 µl of 1% (v/v) Triton X-100/2 mM EDTA/1 mM dithiothreitol/0.1 M phosphate buffer (pH 7.8) for 20 min at room temperature. The total cellular LDH activity, from which the percentage of LDH was calculated, was determined from the activity of the lysate plus the activity of three 80 µl samples previously taken. The LDH activity was determined in quadruplicate in a spectrophotometric kinetic assay by measuring NADH formation in a Molecular Devices (Menlo Park, CA) 96-well plate reader as previously described [17,34,35].

Cell culture and growth inhibition assays

CHO cells (type AA8; ATCC CRL-1859), obtained from the ATCC (Rockville, MD), and DZR cells (a dexrazoxane-resistant CHO-derived cell line) [13,30] were grown in minimum essential medium (α-MEM; Invitrogen, Burlington, Canada) containing 20 mM HEPES (Sigma, St Louis, MO), 100 U/ml penicillin G, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, 10% FBS (Invitrogen) in an atmosphere of 5% CO_2 and 95% air at 37°C (pH 7.4). For the measurement of growth inhibition, cells in exponential growth were harvested and seeded at either 2000 cells/well (CHO) or 6000 cells/well (DZR) in 96-well plates (100 µl/well) and allowed to attach. Control cells were allowed to attach for 24 h prior to treatment with anthracycline for a further 72 h. For the 20-h dexrazoxane pretreatment study the cells were allowed to attach for 3 h and then treated with 100 µM dexrazoxane. After a further 20 h they were then treated with anthracycline for a further 72 h. For the 0.5-h dexrazoxane pretreatment study the cells were allowed to attach for 24 h and then treated with 100 µM dexrazoxane for 0.5 h and then treated with anthracycline for a further 72 h. The cells were preincubated with 100 µM dexrazoxane for 0.5 or 20 h to allow differing times for dexrazoxane

Fig. 2



LDH release from doxorubicin-treated cardiac myocytes is prevented by pretreatment with dexrazoxane. Plot of cumulative percentage LDH release from myocytes that were untreated, treated with 1.3 µM doxorubicin, treated with 100 µM dexrazoxane or treated with 100 µM dexrazoxane and 1.3 µM doxorubicin. In these experiments the dexrazoxane-treated myocytes were pretreated with dexrazoxane for 3 h, doxorubicin was added for a further 3 h and the myocytes were washed with medium containing 100 µM dexrazoxane. Pretreatment of doxorubicin-treated myocytes with 100 µM dexrazoxane reduced the LDH release to levels that were not significantly different than untreated controls. The results were averages from four to eight wells. Where error bars are not seen they are smaller than the size of the symbol.

to hydrolyze to its active metal-chelating form ADR-925 [23]. The inhibition of cell growth was determined by an assay using MTT. The spectrophotometric MTT 96-well plate cell growth inhibition assay, which measures the ability of the cells to enzymatically reduce MTT, has been described [36]. Four replicates were measured at each drug concentration.

Results

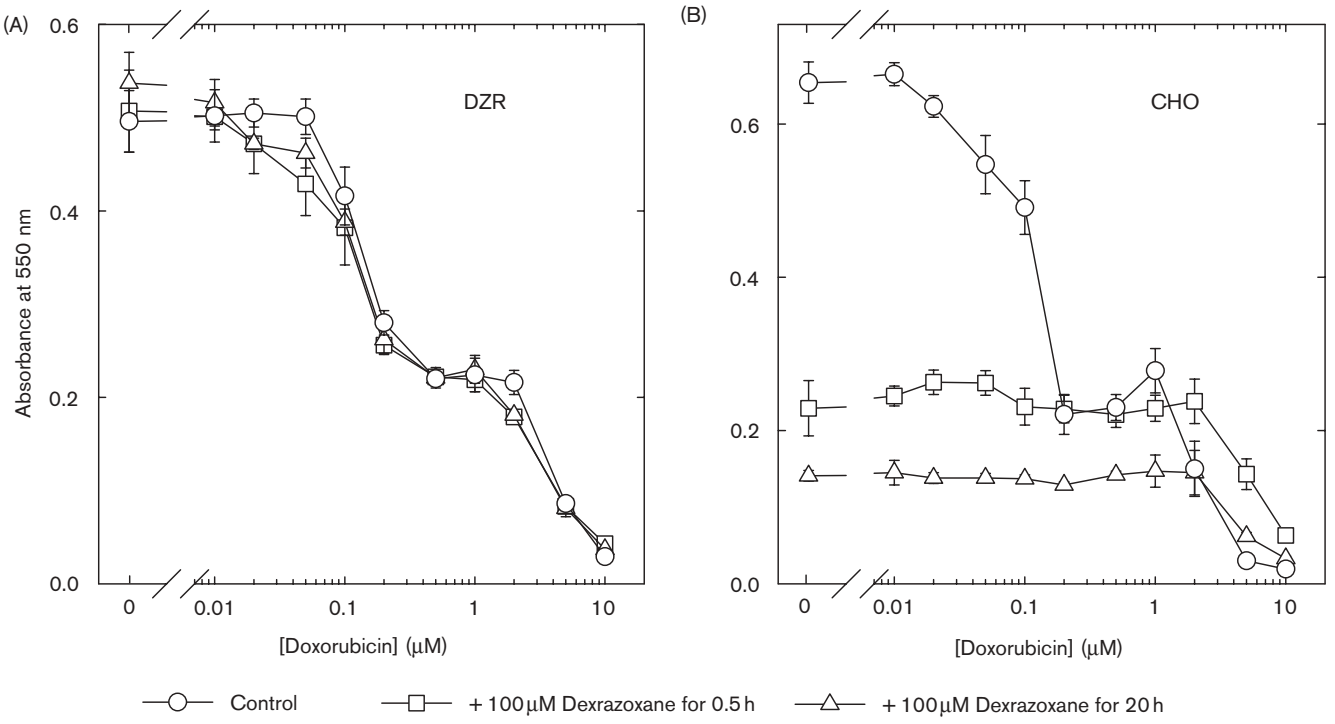
Prevention of doxorubicin-induced LDH release from cardiac myocytes by dexrazoxane

In the results shown in Figure 2, the attached myocytes were pretreated with 100 μM dexrazoxane for 3 h after which doxorubicin (1.3 μM) was added for a further 3 h. The wells were then washed (twice for 20 min) with medium containing 100 μM dexrazoxane and then maintained in this medium for 72 h. The doxorubicin treatment resulted in a significant ($p < 0.003$) increase in the cumulative amount of the LDH released, compared to untreated myocytes at all 3 times. The release of the cytosolic enzyme LDH from myocytes is commonly used as a measure of doxorubicin and other drug-induced damage [37]. The data of Figure 2 also show that pretreatment of the myocytes with 100 μM dexrazoxane for 3 h before doxorubicin treatment resulted in a reduction in doxorubicin-induced LDH release to levels that were not significantly different ($p > 0.08$) from the untreated control values. Continuous incubation of myocytes with dexrazoxane (100 μM) alone did not significantly change ($p > 0.14$) LDH release compared to untreated control levels. The protection against doxorubicin-induced damage is similar to what we previously observed under similar conditions [17,35].

The doxorubicin- and daunorubicin-mediated growth inhibition of dexrazoxane-resistant DZR cells is unaffected by pretreatment with dexrazoxane

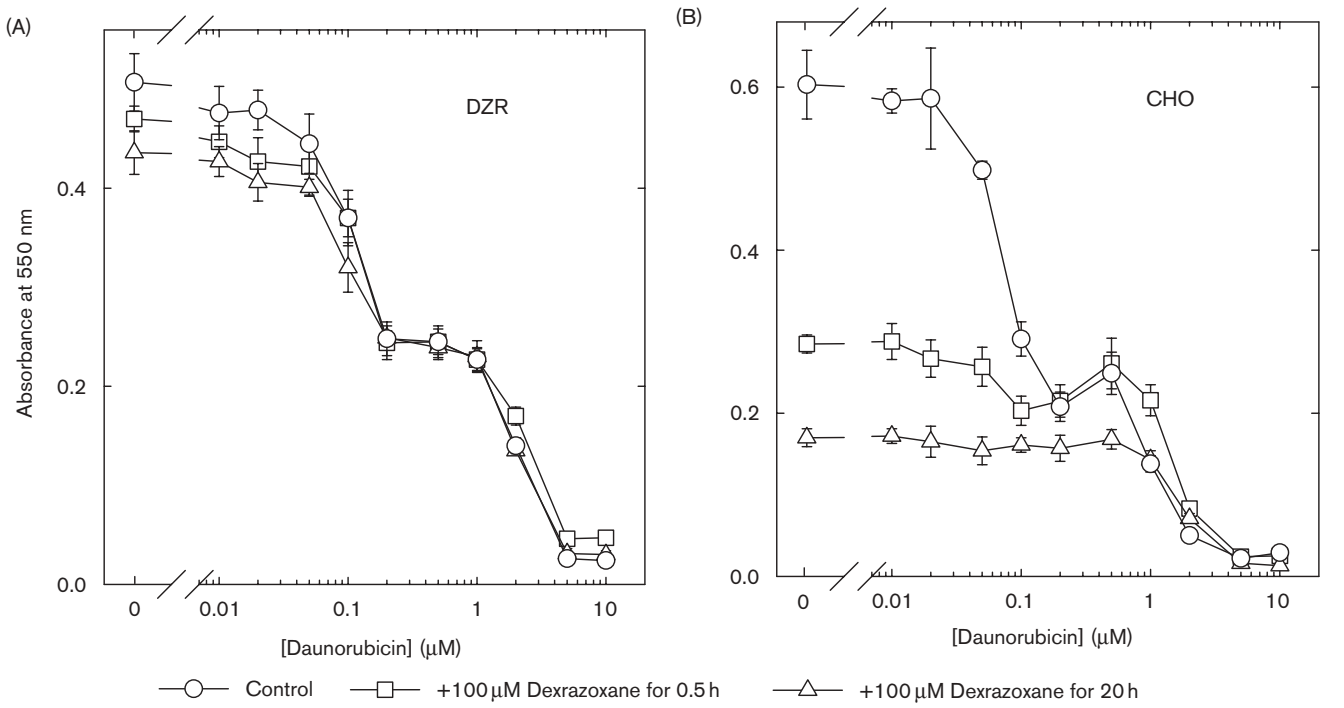
As shown in Figures 3(A) and 4(A), pretreatment of dexrazoxane-resistant DZR cells for either 0.5 or 20 h with 100 μM dexrazoxane had no measurable effect on either doxorubicin- or daunorubicin-mediated growth inhibition. The absorbance values for cells not treated with either anthracycline were also unaffected, indicating that neither dexrazoxane nor its hydrolysis products at this concentration affected the growth of DZR cells. The concentration of 100 μM dexrazoxane in these experiments was chosen as we previously showed that the growth of DZR cells was unaffected by dexrazoxane

Fig. 3



The doxorubicin-mediated growth inhibition of dexrazoxane-resistant DZR cells is unaffected by pretreatment with dexrazoxane. (A) DZR cells were untreated, pretreated with 100 μM dexrazoxane for 0.5 h or pretreated with 100 μM dexrazoxane for 20 h. (B) CHO cells were untreated, pretreated with 100 μM dexrazoxane for 0.5 h or pretreated with 100 μM dexrazoxane for 20 h. After doxorubicin treatment the cells were continuously incubated with both drugs for 72 h and then assayed with MTT. The error bars are SDs from replicates in four wells. Where error bars are not seen they are smaller than the size of the symbol. The absorbance values to the left of the axis break on a linear scale are for cells that were not treated with doxorubicin.

Fig. 4



The daunorubicin-mediated growth inhibition of dexrazoxane-resistant DZR cells is unaffected by pretreatment with dexrazoxane. (A) DZR cells were untreated, pretreated with 100 μM dexrazoxane for 0.5 h or pretreated with 100 μM dexrazoxane for 20 h. (B) CHO cells were untreated, pretreated with 100 μM dexrazoxane for 0.5 h or pretreated with 100 μM dexrazoxane for 20 h. After daunorubicin treatment the cells were continuously incubated with both drugs for 72 h and then assayed with MTT. The error bars are SDs from replicates in four wells. Where error bars are not seen they are smaller than the size of the symbol. The absorbance values to the left of the axis break on a linear scale are for cells that were not treated with daunorubicin.

concentrations up to 500 μM [13]. Above this concentration growth inhibition occurs through depletion of Mg^{2+} and Ca^{2+} from the growth medium by the metal-chelating ADR-925 [13]. As we [12] and others [38,39] have observed before, a partial stimulation of growth was observed in the low micromolar anthracycline concentration range. This effect may be due to the ability of doxorubicin and daunorubicin to inhibit Topo II-mediated DNA cleavage above about 0.5 μM anthracycline concentration [1].

In contrast, as shown in Figures 3(B) and 4(B), the parental CHO cells not treated with either anthracycline showed a large time-dependent decrease in absorbance values after treatment with 100 μM dexrazoxane. We previously showed that dexrazoxane inhibits the growth of the parental CHO cell line with an 50% growth inhibition concentration of 1.8 μM [13].

Discussion

The cardiotoxicity of doxorubicin is thought to be due to the ability of doxorubicin, mediated through the Fe^{3+}–doxorubicin complex, to redox cycle and generate hydroxyl radicals that cause site-specific damage to cell membranes [23,40,41]. Using the myocyte model we previously showed that the clinically approved doxorubicin cardioprotective agent dexrazoxane is able to reduce doxorubicin-induced cytotoxicity in myocytes [17,35]. The concentrations of doxorubicin used in the myocyte model were even smaller (1.3 compared to 12 μM) than those seen clinically at the end of a 60-mg/m^2 doxorubicin infusion period [42]. Thus, the doxorubicin concentrations and the time over which the myocytes were exposed to doxorubicin were in a pharmacologically relevant range.

The DZR cell line with 1500-fold acquired resistance to dexrazoxane [13,30] has a Thr48Ile mutation in Topo IIα [13,30] close to the recently determined dexrazoxane binding site [31,32] and likely sterically interferes with its binding. While the DZR cell line contains one-half the Topo IIα protein that the parent cell line has, its Topo II activity is unchanged [13,30]. The fact that the P-glycoprotein inhibitor verapamil has no effect on the DZR growth-inhibitory effects of dexrazoxane suggests that the primary resistance mechanism is due to the binding site mutation in Topo IIα [13].

Our previous spectrophotometric and HPLC studies [26,27] showed that under physiological conditions dexrazoxane is only slowly hydrolyzed to **B** and **C** ($t_{1/2}$ of 9.3 h at 37°C and pH 7.4), and to the final hydrolysis product ADR-925 ($t_{1/2}$ of 23 h), according to the kinetic scheme shown in Figure 1. Thus, for cells pretreated with dexrazoxane for 20 h, the concentration of ADR-925 in the medium would be about 50 μM at the time the cells were treated with anthracycline. For cells pretreated with dexrazoxane for 0.5 h, the concentration of ADR-925 would originally have been small relative to the dexrazoxane concentration, but by the end of the 72 h incubation period it would be approaching 100 μM. Thus, for either treatment significant amounts of the metal chelating ADR-925 would have been present in the culture medium throughout the course of the experiment. We previously showed that the one-ring open intermediates **B** and **C**, and ADR-925 were over a period of several minutes able to slowly enter cells and displace iron from its complex with calcein [34,43]. However, it is unknown whether sufficient amounts of these metabolites [44] can enter myocytes and bind enough iron to prevent damage. It should be noted that these experiments with dexrazoxane only test for the prevention of iron-based oxygen radical damage, and not damage produced from reduction to the semiquinone free radical and other possible subsequent radical reactions.

In a review [5] of studies conducted at pharmacological concentrations of anthracyclines it was concluded that the primary anti-tumor mechanism was likely through their interaction with Topo II rather than through free radical formation. The differing modes of action on tumors or on the heart may be related to distinct signal transduction mechanisms and survival factors in these tissues [4]. Our results are also in accord with those of a critical review in which it was concluded that generation of free radical species by doxorubicin is not a mechanism of cytotoxicity for most tumor cell lines [7]. Results with the effects of antioxidants on cytotoxicity of anthracyclines towards tumor cells have been inconclusive with some studies reporting protection and others reporting no effect [5,7]. It is of interest to note that a recent study has shown that doxorubicin cardiotoxicity is reduced in mice with a null allele of carbonyl reductase 1 [45]. Carbonyl reductases are thought to be responsible for the conversion of doxorubicin to its more cardiotoxic metabolite doxorubicinol [4,45] that may act in an iron-dependent manner though its perturbation of the aconitase/iron regulatory protein 1 machinery [4]. Thus, in conclusion, the results of this study that showed that dexrazoxane failed to prevent doxorubicin- or daunorubicin-induced inhibition of growth of DZR cells indicates that iron-mediated oxygen radical damage does not contribute to anthracycline-induced growth inhibition of DZR cells.

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