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FREE RADICAL MODES OF CYTOTOXICITY OF ADRIAMYCIN® AND STREPTONIGRIN

WILLIAM DEGRAFF, STEPHEN M. HAHN, J. B. MITCHELL and MURALI C. KRISHNA*

Radiation Biology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

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Abstract—Free radical modes of cytotoxicity of streptonigrin (STN) and Adriamycin® (ADR) in Chinese hamster V79 cells under aerobic conditions were evaluated using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TP), a low molecular weight stable nitroxide free radical with antioxidant properties and desferrioxamine (DF), a transition metal chelator. In addition, exogenous superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), were tested for cytoprotective effects. EPR studies showed that TP reacts with the semiquinones of both ADR and STN and also with O_2^- radicals generated during aerobic redox cycling of the respective semiquinone radicals. Pulsed field gel electrophoresis studies confirmed that DNA double-strand breaks (dsb) induced by STN in V79 cells were inhibited completely by TP, whereas ADR-induced DNA dsb were not affected by TP. Clonogenic cell survival studies showed that STN-induced cytotoxicity could be inhibited completely by DF or TP. Both agents were ineffective in inhibiting ADR-induced cytotoxicity. SOD and CAT were ineffective in protecting against both STN and ADR cytotoxicity. Our results are consistent with a mechanism requiring the semiquinone radical intermediate of STN for cytotoxicity and minimal free radical involvement in ADR-induced V79 cell cytotoxicity.

Key words: nitroxides; Adriamycin; streptonigrin; EPR; semiquinone; free radicals; DNA strand breaks

ADR† is a widely used anticancer agent with significant therapeutic activity [1], though its use is associated with cumulative cardiotoxicity [2]. Several mechanisms have been advanced to explain the antitumor activity of ADR. These include DNA intercalation by the aglycone moiety between adjacent DNA base pairs in the double helix, which results in inhibition of replication, transcription and ultimately translation [3], RNA and protein syntheses [4], stabilization of topoisomerase II–DNA complex, which causes protein-associated DNA strand breaks [5], and free radical mediated cytotoxicity through aerobic redox cycling of the ADR semiquinone radical [6]. ADR and other anthracyclines are also metal chelators with high stability constants for transition metal ions such as iron [7]. Ferric-ADR complexes have been shown to degrade deoxyribose and cleave DNA [8]. Evidence for the generation of hydroxyl radicals and other strong oxidants has also been presented for the ADR–metal complexes [9]. Copper complexes of ADR have been shown to cause oxygen radical dependent lipid peroxidation [10]. ADR activation to the semiquinone radical can be mediated by cellular enzymatic processes [2, 6, 9]. This radical species can subsequently undergo rapid oxidation by molecular oxygen to produce O_2^-, H_2O_2 and other free radical species [2, 11]. Unlike free ADR, ADR covalently bound to DNA was not found to be reduced to the semiquinone radical [12]. Several studies have correlated ADR semiquinone radical formation with antitumor activity. MCF-7 human breast cancer cells resistant to ADR were shown to possess enhanced levels of antioxidants compared with the parent cell line [13, 14]. Moreover, a diminished free radical generation has been proposed to confer cellular resistance to ADR [15]. In addition, the activity of topoisomerase-II in ADR-resistant cells was found to be lower than in the drug-sensitive cells [16].

STN is an aminoquinone antibiotic with demonstrated cytotoxic activity against mammalian cells [17]. Unlike ADR, STN does not intercalate but is known to covalently bind to DNA [18]. STN cytotoxicity has been directly correlated with intracellular reduction of the drug and subsequent free radical mediated DNA degradation [19]. STN covalently bound to DNA has also been suggested to redox cycle *in situ* [20], thereby generating deleterious oxy-radicals in close proximity to DNA.

Cyclic nitroxides are stable radicals that selectively react with paramagnetic, though not diamagnetic species. Consequently nitroxides not only serve as radical chain breakers and effective cytoprotective agents, but also act as sensitive probes for monitoring free radical mediated processes. Nitroxides are non-mutagenic, non-toxic free radicals [21] with SOD-mimetic activity [22], which are also efficient scavengers of reactive free radicals [23, 24]. Such properties led to a screening for its antioxidant effects *in vitro* and *in vivo*. TP protected Chinese

* Corresponding author: Dr. Murali C. Krishna, Building 10, Room B3 B69, NIH, Bethesda, MD 20892. Tel. (301) 496-7511; FAX (301) 480-2238.

† Abbreviations: ADR, Adriamycin®; STN, streptonigrin; TP, Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; DF, desferrioxamine, Desferal; SOD, superoxide dismutase; CAT, catalase; dsb, double-strand breaks; NADH, β-nicotinamide-adenine dinucleotide, reduced; DETAPAC, diethylenetriamine pentaacetic acid; and PFGE, pulsed field gel electrophoresis.

hamster V79 cells against damage induced by exposure to O_2^-, H_2O_2, *t*-butylhydroperoxide, X-rays and mitomycin C [25–28]. Post-ischemic reperfusion injury of rat hearts was prevented by a lipid soluble analog of TP [29], and TP also provided whole body radioprotection of mice against the lethal effects of ionizing radiation [30]. In the present study, Tempol (TP) was used as a specific probe for free radical mediated cytotoxicities of STN and ADR using EPR spectroscopy, PFGE, and clonogenic cell survival assays. PFGE has been shown to be a sensitive, specific assay for DNA dsb [31]. PFGE yields data on the frequency of induction of DNA dsb quantitatively similar to that found by neutral filter elution or other techniques of DNA dsb measurement [32–34]. Olive [35] have pointed out that neutral filter elution and sedimentation assays are sensitive not only to the integrity of the DNA molecules, but also to their conformation. Indeed, in V79 cells, PFGE appears to be a more sensitive measure of radiation-induced dsb than neutral filter elution [36], showing little or no dependence of chromatin structure. The results from these studies indicate that DF and TP were effective in inhibiting STN-induced V79 cell cytotoxicity, whereas ADR-induced cytotoxicity remained unaffected. Our results suggest TP to be effective in inhibiting cellular damage induced by redox cycling quinones. Lack of protective effects of TP and DF suggests a minimal role of free radical modes of cytotoxicity induced by ADR in V79 cells.

MATERIALS AND METHODS

Chemicals. SOD and NADH dehydrogenase (EC 1.6.99.3) were purchased from the Sigma Chemical Co. (St. Louis, MO); xanthine oxidase, CAT and NADH were obtained from the Boehringer Mannheim Co. (Anaheim, CA); TP was purchased from the Aldrich Chemical Co. (Milwaukee, WI) and used without purification. Desferal (DF) was a gift from CIBA GEIGY (Nutley, NJ). Streptonigrin was from Sigma and Adriamycin was from Adria Laboratories (Columbus, OH).

Electron paramagnetic resonance. For EPR experiments, samples were drawn into gas permeable Teflon capillary tubes (Zeus Industries, 0.8 mm i.d., 0.5 mm wall thickness). Each capillary was folded twice, inserted into a narrow quartz tube (2.5 mm i.d) open at both ends, and then placed in the EPR cavity. During the experiment, gases of desired compositions were flushed around the sample while the tube remained undisturbed in the cavity. EPR spectra were recorded on a Varian E-109 X-band spectrometer operating at 9.45 GHz with a field modulation frequency of 100 kHz and modulation amplitude of 0.5 G and 20 mW microwave power.

Cell culture. Chinese hamster V79 cells were grown in F12 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Survival was assessed in all studies by the clonogenic assay. The plating efficiency ranged between 80 and 90%. Stock cultures of exponentially growing cells were trypsinized, rinsed, and plated (5×10^5 cells/dish) into a number of 100-mm Petri dishes and incubated for 16 hr at 37° prior to experimental protocols. Drug (ADR or STN) was added to exponentially growing cells in complete F12 medium (final concentrations of ADR or STN were in μg/mL) in the presence or absence of TP (10 mM). TP treatment at these concentrations (10 mM) did not alter the plating efficiency. Immediately after treatment, cells were rinsed, trypsinized, counted, and plated for macroscopic colony formation. SOD and CAT were added immediately before drug treatment, whereas DF was added 2 hr prior to drug exposure. Final concentrations of DF, SOD, and CAT were 500 μM, 100 μg/mL, and 100 U/mL, respectively. DF, SOD, or CAT treatment did not alter the plating efficiency. For each concentration determination, cells were plated in triplicate, and each experiment was performed a minimum of two times. After treatment, plates were incubated for 7 days, and then colonies were fixed with methanol/acetic acid (3:1) and stained with crystal violet. Colonies containing >50 cells were scored. Error bars shown in figures represent standard error of the mean and are shown when larger than the symbol.

In experiments requiring exposure to STN under hypoxic conditions, cells were dispersed in 1.8 mL of medium, plated (2.5×10^5) into specially designed glass flasks, and incubated at 37° overnight. TP (10 mM) was added to the cell monolayer immediately before the gassing procedure. The flasks were then sealed with soft rubber stoppers, and 19-gauge needles were pushed through the rubber stopper to provide entrance and exit ports for a humidified gas mixture of 95% nitrogen and 5% CO_2 (Matheson Gas Products). Each flask was also equipped with a ground-glass side arm vessel which, when rotated and inverted, could deliver 0.2 mL of medium containing STN. Stoppered flasks were connected in series, mounted on a reciprocating platform, and gassed at 37° for 60 min. This gassing procedure results in an equilibrium between the gas and liquid phase (in both the medium over the cell monolayer and in the solution in the sidearm) and yielded oxygen concentrations in the effluent gas phase of <10 ppm as measured by a Thermox probe. After 60 min of gassing, the hypoxic drug solution in the side arm was added to the cell monolayer culture. The cells were exposed to STN for 1 hr under hypoxic conditions. Cell survival after drug exposure was assessed as described above. For some experiments, DF, SOD, and CAT were added to the cell monolayer immediately before the gassing procedure to final concentrations of 500 μM, 100 μg/mL, and 100 U/mL, respectively. DF, SOD, or CAT treatment did not alter the plating efficiency.

Preparation of DNA for electrophoresis. Cells for electrophoresis were plated as described above, and the DNA was labeled by incubating the cells with 0.02 μCi [^{14}C]thymidine/mL for 24 hr prior to irradiation or drug exposure. DNA was prepared for electrophoresis by the methods of Schwartz and Cantor [37] and Gardiner *et al.* [38] as modified by Ager and Dewey [34] and Stamato and Denko [31] After STN or ADR exposure, the cells were trypsinized, rinsed, and resuspended in PBS at 10^7 cells/mL. An equal volume of 1% low gelling temperature agarose was added, and the cell suspension was drawn into 3/32 inch (i.d.) silicone

tubing with a syringe. Both ends of the tubing were clamped, and the tubing was immersed in an ice bath to rapidly solidify the agarose. The agarose was then extruded from the tubing, cut into 5 mm lengths, and these "plugs" were placed into 1.5-mL centrifuge tubes. This procedure results in approximately 10^5 cells/5 mm plug. DNA was purified by incubating at 55° in ESP buffer (0.5 M EDTA, 1% Sarkosyl, and 1 μg/mL proteinase K) for 24 hr. The plugs were then rinsed in TE buffer (10 mM Tris, 1 mM EDTA) for 24 hr with three buffer changes. RNA was digested by incubation with 0.1 μg/mL boiled RNase A in TE buffer for 2 hr at 37°.

Electrophoresis conditions. Agarose gels (0.8%) were cast in 0.5× TBE (1× TBE = 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA), loaded into 2 × 6 × 5 mm wells, and the wells were sealed with melted agarose. Electrophoresis was carried out for 24 hr at 56 V (4 V/cm), with a 3:1 ratio of forward to reverse pulse time. The initial forward pulse time was 7.5 sec (reverse pulse 2.5 sec), increasing to a final forward pulse time of 90 sec (final reverse pulse 30 sec). The running buffer (0.5× TBE) was recirculated and cooled to maintain a temperature of 12–15°. These electrophoresis conditions were chosen based on methods of Stamato and Denko [38], and the desire to keep the released DNA concentrated in a narrow band to facilitate quantitation (see below).

After electrophoresis, the gels were soaked in 0.5 μg/mL ethidium bromide for 30 min, destained with distilled water for 30 min, and photographed on a UV light box. The lanes were separated from one another, and the well containing the plug was separated from the portion of the lane containing the released DNA. These small pieces of agarose were put into separate scintillation vials, and the agarose was melted by placing each vial on a hot plate, after adding 50 μL of concentrated HCl to prevent resolidification of the agarose. Hydroflour® (15 mL; National Diagnostics) was added to each vial, and radioactivity was determined by counting on an LKB 1217 scintillation counter. The data are expressed as "% DNA remaining in the well" and are calculated as follows:

$$\%\ \text{DNA remaining} = \frac{\text{cpm in the well}}{\text{cpm in the lane} + \text{cpm in the well}} \times 100$$

At least two independent experiments were run for each treatment, with duplicate plugs run at each concentration level or control.

RESULTS

Reactions with TP. The reactions of the respective semiquinones of ADR and STN with Tempol in the presence and absence of oxygen were studied using EPR spectroscopy. The semiquinone radicals were generated by exposing STN or ADR at 100 μM to NADH dehydrogenase in the presence of NADH as the cofactor. Figure 1A shows the change in the EPR signal intensity of Tempol (100 μM) as a function of time in the presence of STN or ADR

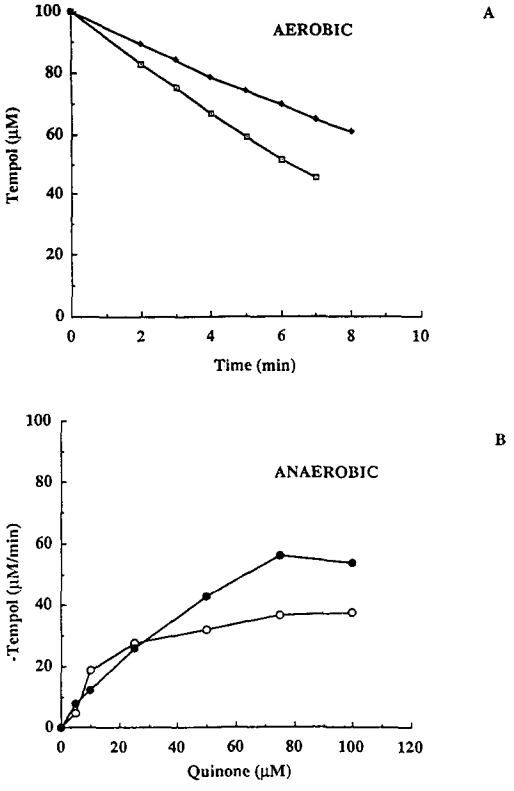

Fig. 1. (A) EPR signal intensity of Tempol as a function of time in the reaction of aerobic NADH dehydrogenase (0.2 U/mL), NADH (2 mM), Tempol (100 μM) in phosphate-buffered saline (50 mM, pH 7.4) containing DETAPAC (100 μM) and catalase (200 U/mL) in the presence of 100 μM STN (filled symbols) or 100 μM ADR (open symbols). No reduction was observed when the reaction was conducted in the absence of either of the quinones. (B) Reduction rate of Tempol under anaerobic conditions, in the NADH dehydrogenase/NADH reaction in the presence of various concentrations of STN (filled circles) or ADR (open circles). The reaction also contained SOD (250 U/mL), CAT (200 U/mL) and DETAPAC (100 μM).

and NADH dehydrogenase/NADH reaction in air-saturated phosphate buffer (50 mM, 7.4) and NADH (2 mM). CAT (200 U/mL) and DETAPAC (100 μM) were also included in the reaction to prevent any contribution from adventitious transition metals reacting with H_2O_2. As can be seen, the signal intensity of Tempol decreased as a function of time. This reduction in intensity was prevented by the addition of SOD. The loss of this signal is attributable to the formation of the hydroxylamine (Tempol-H) based on the observation that after the completion of the reaction, addition of the one-electron oxidizing agent, ferricyanide, completely restored the signal to its original level. Therefore, superoxide generated by the semiquinone radicals of either STN or ADR is responsible for the reduction of Tempol to Tempol-H. Increasing the concentration of the quinone (STN or ADR) resulted in enhanced production of

superoxide, as indicated by an enhanced loss of the Tempol EPR signal.

In anaerobic conditions, Tempol (100 μM) was exposed to ADR or STN in the NADH dehydrogenase/NADH reaction and the reduction of Tempol was followed by EPR spectroscopy (Fig. 1B). In the absence of quinone, there was no reduction, whereas in the presence of various concentrations STN or ADR, Tempol was reduced as a function of time. The rate of reduction was dependent on the concentration of the quinone. That the reduction proceeded by one-electron was confirmed by the observation that, after the completion of the reaction, when ferricyanide was added, the EPR signal intensity was completely restored to its original level. Since TP also rapidly penetrates into cells through passive diffusion [25], it can be anticipated that if either drug (STN or ADR) causes cytotoxicity by free radical reactions in intracellular regions, then TP should potentially inhibit damage at several levels which include (a) dismutation of superoxide [22], (b) oxidation of reduced transition metal ions [24, 25] (3) oxidation of the semiquinone radical [28], and (d) inhibition of lipid peroxidation by breaking the free radical chain reactions [24].

DNA strand break assay. Stamato and Denko [31] have shown that field inversion gel electrophoresis is specific for measurement of DNA dsb, which correlate with cell death. DNA dsb were detected by this technique in V79 cells exposed to STN or ADR. Figure 2 shows the ethidium bromide stained field inversion electrophoresis gel of DNA from V79 cells exposed to STN in the absence or presence of TP. In the absence of TP, extensive DNA dsb could be detected from V79 cells exposed to STN (Fig. 2, top). However, when the cells were exposed to STN in the presence of TP (10 mM), little if any DNA dsb could be detected in the lower half of the gel (Fig. 2, bottom). Figure 3 shows an ethidium bromide stained gel of cells treated with ADR in the presence or absence of TP (10 mM). The percent DNA remaining in the well from the STN- and ADR-treated V79 cells was quantitated by counting radioactively labeled DNA released into the gel and that remaining in the well. Panels A and B of Fig. 4 show plots from STN- and ADR-treated V79 cells, respectively. TP completely inhibited DNA release in STN-treated V79 cells (Fig. 4A), whereas DNA release from ADR-treated V79 cells was unaffected by the presence of TP (Fig. 4B).

Cellular cytotoxicity. The modulation of STN- and ADR-induced cytotoxicity in Chinese hamster V79 cells by SOD, CAT, DF and TP has been studied by clonogenic cell survival. The concentrations of SOD, CAT, DF and TP were chosen based on earlier observations [25] where complete protection was observed when V79 cells were exposed to O_2^- and H_2O_2 directly. Figure 5A shows the effects of SOD, CAT, DF and TP on V79 cell cytotoxicity when treated with STN under aerobic conditions. A concentration-dependent cytotoxicity was observed in V79 cells when treated with STN for 1 hr aerobically. The presence of SOD (100 μg/mL) or CAT (100 U/mL) failed to inhibit STN-induced cytotoxicity at all concentrations of the drug tested. Pretreatment of cells with DF (500 μM) prior to STN exposure completely inhibited the cytotoxicity. STN-induced cytotoxicity was also inhibited when V79 cells were exposed to the drug in the presence of TP (10 mM). Hypoxic exposure of V79 cells to various concentrations of STN for 1 hr also caused a concentration-dependent decrease in clonogenic viability (Fig. 5B). Similar to results observed under aerobic conditions, pretreatment of cells for 2 hr with DF (500 μM) or the simultaneous presence of TP (10 mM) completely inhibited STN-induced hypoxic cytotoxicity. To determine whether TP inhibits the cytotoxicity by inhibiting the uptake of cellular STN, cells were treated with STN under hypoxic conditions for 1 hr. On readmitting air after hypoxic incubation with STN, significant cytotoxicity was observed. However, the presence of TP (10 mM) at the time of reexposure to air after hypoxic incubation completely abated the STN-induced cytotoxicity (data not shown). This observation suggests that TP provides protection even after drug uptake and subsequent bioreductive activation events have occurred. As expected, SOD and CAT had little or no effect on V79 cell survival when exposed to STN under hypoxic conditions.

ADR-induced cytotoxicity in V79 cells and the effects of TP, DF, SOD, and CAT on ADR-induced cytotoxicity are presented in Fig. 6. SOD and CAT failed to inhibit ADR-induced cytotoxicity at all concentrations tested. Pretreatment with DF (500 μM) prior to ADR exposure also had no effects on cell survival (Fig. 6A). Although TP reacts with ADR semiquinone and can also catalytically dismutate O_2^- generated by redox cycling of ADR semiquinone, no protective effects of this agent (10 mM) on the survival of ADR-treated V79 cells were observed (Fig. 6B).

DISCUSSION

STN and ADR are quinone based agents that can undergo cellular flavoenzyme catalyzed one-electron reduction to produce the respective semiquinone radicals [6, 9, 39]. In aerobic conditions, the semiquinone radicals participate in redox cycling to generate O_2^- and H_2O_2, which, in the presence of transition metal ions, can induce damage presumably by site specific generation of ·OH radicals [2, 40, 41]. Intracellular generation of semiquinone radicals leading to DNA degradation has been proposed to explain the oxygen-dependent antibacterial effects of STN [19, 42]. ADR is a multifunctional agent that mediates cellular cytotoxicity through several pathways. It can rapidly localize in the nucleus of mammalian cells, especially in the chromatin [1]. Semiquinone radical generation from ADR by cellular flavoenzymes and subsequently redox cycling to produce O_2^- and H_2O_2 have been investigated thoroughly [6, 9, 39, 43]. Several reports present EPR spectroscopic evidence for the formation of the ADR semiquinone radicals in mammalian cells exposed to ADR under hypoxic conditions [15, 44]. Under aerobic conditions, DMPO-spin trapping coupled with EPR detection provided information supporting the production of reactive oxygen radicals (O_2^- and ·OH) during enzymatic and cellular

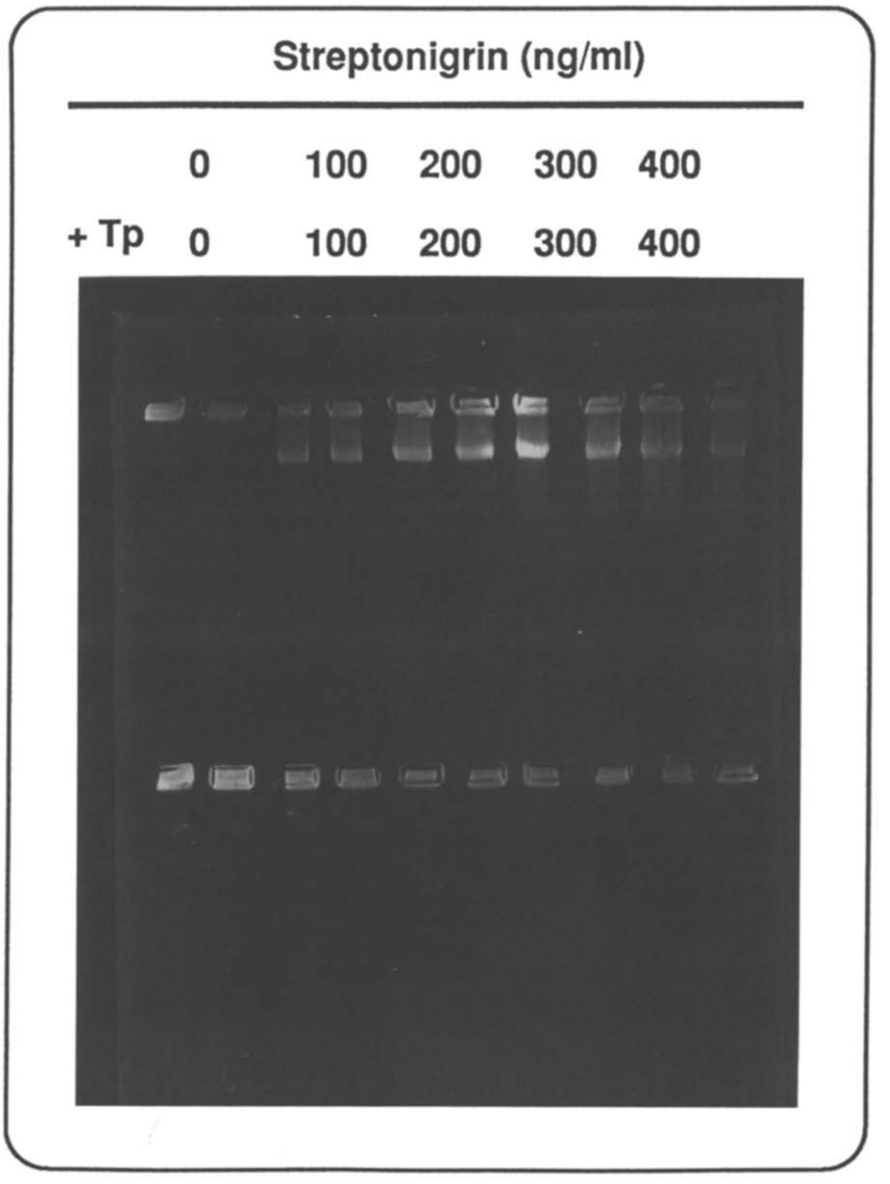

Fig. 2. Field inversion electrophoresis gel of DNA extracted from V79 cells treated with 0–400 ng/mL streptonigrin in the absence (top) or presence (bottom) of 10 mM TP, and stained with ethidium bromide.

incubations of ADR under aerobic conditions [41]. Furthermore, several independent investigations utilizing ADR-sensitive and ADR-resistant cell lines have revealed that (i) drug-resistant cells have increased levels of antioxidant enzymes [13, 14, 45], (ii) depletion of thiols in ADR-resistant cells reverses the drug resistance [46], and (iii) ADR-resistant cells are cross-resistant to other modes of oxidative damage including superoxide, H_2O_2, and hyperoxia but not to ionizing radiation [45]. Based on these reports, increased free radical detoxifying processes, in addition to increased efflux of the drug [47], have been suggested as cellular mechanisms of ADR resistance. Other processes that do not involve free radical intermediates in the ADR cytotoxicity are (i) DNA strand breaks due to the anthracycline-induced stabilization of topoisomerase II-DNA complex [5] and (ii) intercalation of the aglycone moiety between adjacent base pairs of DNA resulting in inhibition of replication as well as RNA and protein syntheses [3, 4]. In these latter cases, TP may be expected to provide cytoprotection.

Protection by SOD and CAT. Our previous studies in V79 cells [25] have shown that CAT and TP completely inhibited the O_2^- and H_2O_2-induced cytotoxicity, whereas SOD was not effective. In the same study, preincubation of cells with DF (500 μM) completely inhibited the O_2^- and H_2O_2-induced cytotoxicity. The lack of protection of V79 cells treated with ADR or STN by exogenous SOD and

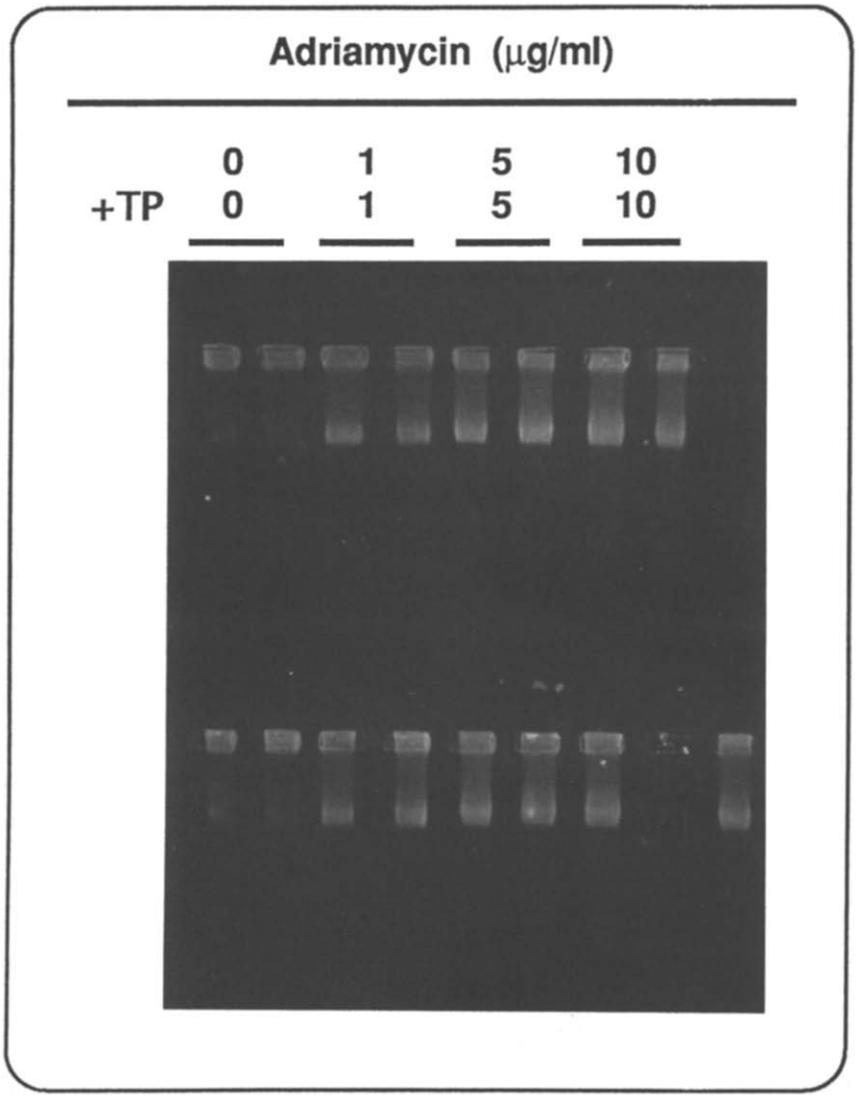

Fig. 3. Field inversion electrophoresis gel of DNA extracted from V79 cells treated with 0–10 μg/mL ADR in the absence (top) or presence (bottom) of 10 mM TP, and stained with ethidium bromide.

CAT suggests that the damage induced by both of these agents is predominantly intracellular since SOD and CAT, though efficient in detoxifying O_2^- and H_2O_2, respectively, are restricted to the extracellular space and hence may not afford protection against damage induced by intracellular oxidants. Earlier studies with Ehrlich tumor cells [40] have shown that ADR cytotoxicity was partially blocked by SOD, CAT and DF. The difference in the results obtained in this study from those seen in V79 cells may be due to different cell types and also different biologic end points used.

Protection by DF. DF is an efficient chelator of metal ions and has been used to inhibit oxidative damage mediated by transition metal ions. Inhibition of damage induced by O_2^-, H_2O_2, and organic hydroperoxides in V79 cells preincubated with DF (500 μM) has also been observed [25]. In this study, pretreatment of V79 cells with DF completely inhibited STN-induced aerobic cytotoxicity (Fig. 5); however, no protection of ADR-induced V79 cell toxicity was observed by pretreatment with DF. In a recent report, DNA base modification has been observed by aerobic incubations of ADR and flavoenzymes with isolated human chromatin; added transition metal ions (iron and copper) potentiate this effect [43]. The data were found to be consistent with the redox cycling of ADR to generate typical hydroxyl radical induced base modifications. Further evidence was provided by the significantly lower levels of DNA base modification by 5-iminodaunorubicin, which is the non-redox cycling analog of ADR [43]. However, in the present study with intact V79 cells exposed to STN and ADR, the complete protection of cytotoxicity by DF in the case of STN and the lack of protection in ADR-treated cells suggest that

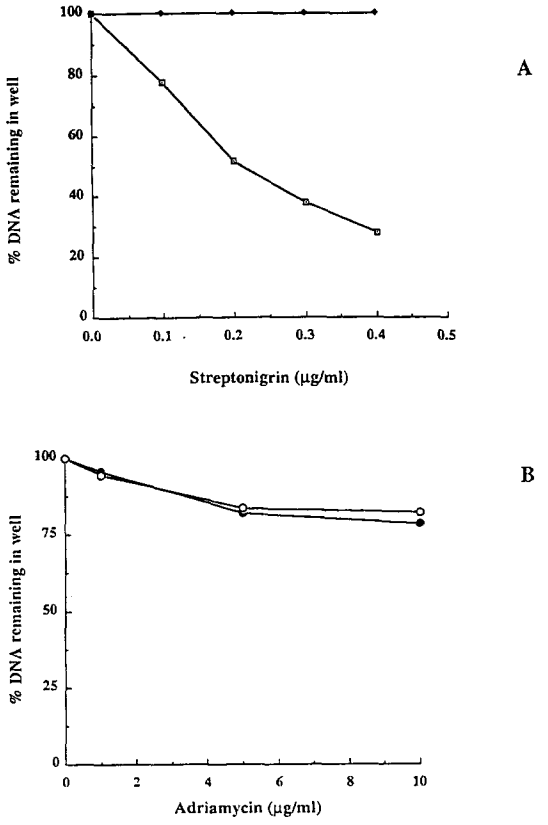

Fig. 4. (A) Percent DNA remaining in the well from STN-treated V79 cells, determined by counting radioactively labeled DNA released into the gel and that remaining in the well. Open symbols represent STN exposure in the absence of Tempol, and closed symbols represent STN exposure in the presence of Tempol. (B) Percent DNA remaining in the well from ADR-treated V79 cells, determined by counting radioactively labeled DNA released into the gel and that remaining in the well. Closed symbols represent ADR exposure in the absence of Tempol, and open symbols represent ADR exposure in the presence of Tempol.

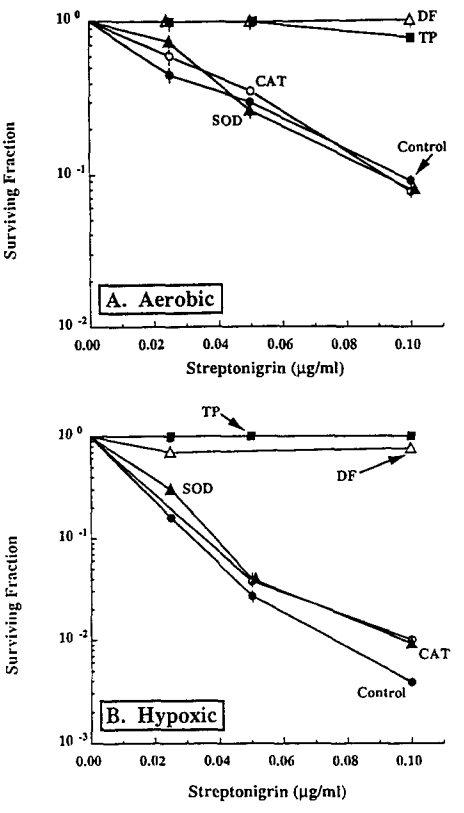

Fig. 5. (A) Concentration–response curves for Chinese hamster V79 cells exposed to various concentrations of STN under aerobic conditions in the absence and presence of SOD (100 μg/mL), CAT (100 U/mL), DF (500 μM) and TP (10 mM). (B) Concentration–response curves for Chinese hamster V79 cells exposed to various concentrations of STN under hypoxic conditions in the absence and presence of SOD (100 μg/mL), CAT (100 U/mL), DF (500 μM) and TP (10 mM).

transition metal ions are involved in the cytotoxicity of STN but not in the case of ADR.

Protection by TP. TP is an uncharged, hydrophilic stable nitroxide that penetrates cell membranes readily [25]. In earlier studies, TP has been shown to be non-toxic and non-mutagenic to mammalian cells exposed to a 10 mM concentration for several hours [21]. Antimutagenic effects of TP were observed in mammalian cells exposed to ionizing radiation, H_2O_2 and neocarzinostatin [21]. It has been shown to be an effective mimic of SOD [22] and to protect V79 cells against damage induced by $O_2^{\cdot-}$, H_2O_2 and organic hydroperoxides [25]. Reactions with reactive radicals such as alkyl, alkoxyl and alkyl peroxyl radicals and termination of free radical chain reactions have been proposed to be the chemical basis for its inhibition of oxidative cellular damage [23, 24]. Nitroxides also represent a new class of aerobic radioprotectors *in vitro* and *in vivo* [27, 30, 48]. They have been shown to maintain transition metal ions in the oxidized condition, thereby potentially interrupting metal-catalyzed decomposition of H_2O_2 to produce the highly oxidizing ·OH radical [25]. Inhibition of hypoxic cytotoxicity of mitomycin C has been attributed to its reaction with the semiquinone radical form of the drug [28]. In addition, TP protects beating cardiomyocytes against H_2O_2-induced damage [49], and a lipophilic derivative of TP has been shown to inhibit post-ischemic reperfusion damage in rat hearts [29]. The efficacy of TP to protect biological systems against diverse types of free radical mediated oxidative damage prompted the current study in which V79 cells were treated with multifunctional drugs such as ADR. In addition, STN was included in this study since it mediates cytotoxicity through the redox cycling of the semiquinone radical intermediate. Thus, it can serve as a model system to screen the efficacy of TP and other nitroxides in protecting mammalian cells against oxidative damage induced by oxygen radicals generated by redox cycling of the semiquinone radical. Currently, we are evaluating the ability of a number of nitroxide

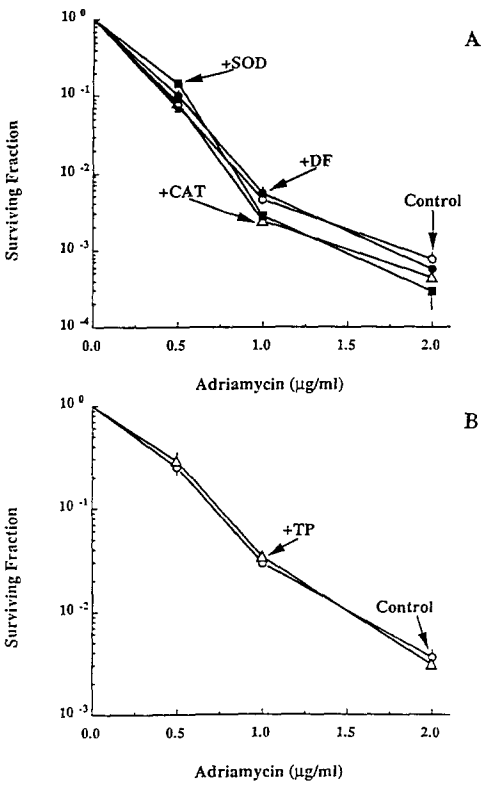

Fig. 6. (A) Concentration–response curves for Chinese hamster V79 cells exposed to various concentrations of ADR in the absence and presence of SOD (100 μg/mL), CAT (100 U/mL), and DF (500 μM). (B) Concentration–response curves for Chinese hamster V79 cells exposed to various concentrations of ADR in the absence and presence of TP (10 mM).

derivatives with different physico-chemical properties to provide protection against oxidative damage induced by different modalities including redox cycling quinones such as STN.

STN-induced DNA dsb in V79 cells were inhibited completely when the incubations were carried out in the presence of TP (10 mM). Studies on the survival of V79 cells show that the presence of TP provided significant protection against STN cytotoxicity. The inhibition by TP of STN-induced V79 cell cytotoxicity could be explained in terms of (i) catalytic dismutation of O_2^-, (ii) oxidation of reduced transition metals, (iii) oxidation of semiquinone radicals, and (iv) termination of free radical chain reactions.

ADR also induces DNA dsb as detected by PFGE. However, the presence of TP had negligible effects on ADR-induced DNA dsb. Cell survival analysis indicates similar behavior, i.e. TP had little or no effect on V79 cell survival when the cells were treated with ADR. The ineffectiveness of TP to protect against ADR-induced cytotoxicity in spite of its rapid reaction with O_2^- and the semiquinone radical of ADR suggests that ADR causes V79 cell cytotoxicity with minimal contribution from free radical pathways originating from the semiquinone intermediate.

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