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Role of NAD(P)H:Quinone Oxidoreductase (DT-diaphorase) in Cytotoxicity and Induction of DNA Damage by Streptonigrin

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ABSTRACT. The metabolism, cytotoxicity, and genotoxicity of streptonigrin (SN) were determined in two human colon carcinoma cell lines: HT-29 with high NAD(P)H:quinone oxidoreductase (EC 1.6.99.2, DTD) activity and BE with undetectable DTD activity. Dicumarol-sensitive oxidation of NADH was observed with HT-29 cytosol, but not with BE cytosol. Oxygen consumption was also observed using HT-29 cytosol, but was absent with BE cytosol. Dicumarol inhibited oxygen consumption with HT-29 cytosol, but deferoxamine had no effect, suggesting that divalent metal cations were not necessary for efficient auto-oxidation of SN hydroquinone. In cytotoxicity studies, SN was much more toxic to the DTD-rich HT-29 cells than to the DTD-deficient BE cells. Deferoxamine decreased toxicity in both cell lines, implicating hydroxyl radicals produced during Fenton-type reactions as the toxic species. In the genotoxicity assay, SN induced a much higher incidence of DNA strand breaks in HT-29 cells than in BE cells, and deferoxamine protected against DNA strand breaks in both cell lines. Some evidence of DNA repair was also observed in the two cell lines. These results support an important role for DTD in the cytotoxicity and genotoxicity of SN in the high DTD HT-29 colon carcinoma cell line. BIOCHEM PHARMACOL 51;5:645–652, 1996.

KEY WORDS. DT-diaphorase; streptonigrin; cytotoxicity; genotoxicity; bioreductive activation; DNA strand breaks

SN¶ is an antitumor antibiotic from *Streptomyces flocculus* [1] that has antitumor activity against a broad range of tumors [2–4]. Clinical use of SN has been limited by reports of delayed myelotoxicity [5–7], but positive results have been reported for SN both as a single agent [8, 9] and in combination therapy [10, 11].

SN is a phenyl-pyridylquinoline with an aminoquinone moiety and multiple metal complexation sites [12]. Formation of SN–metal–DNA complexes has been demonstrated [13–15], but neither covalent binding nor intercalation appears to be involved in the interaction [16]. SN has been shown to inhibit synthesis of both DNA and RNA [17], to cause single-strand cleavage of DNA after reduction by NADH [16], to cause extensive chromosomal breakage and abnormalities *in vivo* [18], and to inhibit topoisomerase II [19]. SN requires reductive activation and the presence of oxygen to produce its DNA-damaging effects [16]. Activation of SN can be accomplished via either a one- or two-electron reductase to produce a semiquinone radical or hydroquinone, respectively. Either form can react with molecular oxygen to produce reactive oxygen species and regeneration of the parent quinone. In the presence of metal ions, hydroxyl radicals may be produced via a Fenton-type reaction. These radicals are believed to be responsible for the DNA degradation [16, 20].

Bachur *et al.* [21] demonstrated that SN can be reduced to the semiquinone by purified P450R, a one-electron reductase, with subsequent production of superoxide. Lown and coworkers [16] showed that the hydroquinone of SN could be generated by reduced pyridine nucleotides in a two-electron reduction. In this case, reduction led to single-strand cleavage of DNA, a process that was enhanced by cuprous and ferrous ions and inhibited by superoxide dismutase, catalase, and free radical scavengers. DTD is a flavoprotein that catalyzes the two-electron reduction of quinones. It is unique for its ability to utilize either NADH or NADPH as an electron source, and it can be inhibited by dicumarol [22]. Although DTD is generally known for its detoxifying properties [23, 24], it may also

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¶ *Abbreviations:* SN, streptonigrin; DTD, NAD(P)H:quinone oxidoreductase (EC 1.6.99.2); MC, mitomycin C; AZQ, diaziquone; MeDZQ, 2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone; P450R, NADPH:cytochrome P450 oxidoreductase; B5R, NADH:cytochrome b_5 oxidoreductase; XO, xanthine oxidase; XDH, xanthine dehydrogenase; DCPIP, 2,6-dichlorophenol-indophenol; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; and IC_{50}, concentration at which cell survival equals 50% of control.

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function as an activating enzyme for antitumor agents requiring bioreductive activation. MC [25], AZQ [26] and its analog MeDZQ [27], the indoloquinone E09 [28], and the dinitrophenylaziridine CB 1954 [29] can be bioactivated by DTD, and we have shown recently that SN is an excellent substrate for both rat and human recombinant DTD [30, 31].

Elevated levels of DTD or DTD mRNA have been found in tumors or tumor cell lines from the lung [32, 33], liver [32, 34], colon [32], breast [32, 35], and brain [36]. DTD levels have been shown to be elevated markedly in NSCLC relative to SCLC and normal lung [33]. We demonstrated recently that there was a good correlation between DTD activity and cytotoxicity of MC and MeDZQ in a panel of lung and breast cancer cell lines [31]. In the same report, E09 and SN were found to have the greatest selective toxicity to a lung cancer cell line with high DTD activity versus a lung cancer cell line with undetectable DTD activity [31]. An enzyme-directed approach to cancer chemotherapy that targets tumors with high DTD activity using bioreductive antitumor agents that are efficient substrates for DTD has the potential for improving selectivity and decreasing toxicity.

In this report, we demonstrate that SN is preferentially genotoxic and cytotoxic to DTD-rich HT-29 human colon carcinoma cells versus DTD-deficient BE human colon carcinoma cells. We also present evidence for hydroxyl radical ($OH^{\bullet}$) mediated DNA single-strand breaks in HT-29 cells following exposure to SN.

MATERIALS AND METHODS

Materials

SN, DCPIP, dicumarol, deferoxamine, NADH and NADPH were obtained from the Sigma Chemical Co., St. Louis, MO. All other reagents were at least of analytical grade.

Cell Culture

HT-29 and BE human colon carcinoma cells were maintained at 37° under an atmosphere of 95% air/5% CO_2. The cells were grown as monolayers in Eagle's minimum essential medium supplemented with 10% bovine calf serum, 2 mM L-glutamine, and gentamicin or penicillin/streptomycin.

Cell Cytosol Preparation

Cell cytosol from HT-29 and BE cells was obtained from six 100-mm tissue culture dishes. Cells were grown to 80% confluence and washed with PBS. The cells were trypsinized, medium was added to inactivate the trypsin, and the cells were pelleted by centrifugation at 4° in 15 mL conical tubes. The cell pellets were washed twice with PBS followed each time by centrifugation. The cell pellets were suspended in ice-cold buffer containing 25 mM Tris-HCl and 125 mM sucrose (pH 7.4). The suspensions were probe sonicated for 30 sec on ice and centrifuged at 100,000 *g* for 1 hr at 4° to yield a clear cytosolic fraction.

HPLC Analysis

Reduction of SN was followed by HPLC using an Alltech C18 (5 μm, 250 mm × 4.6 mm) column with a Shimadzu HPLC system (SCL-6A controller, SPD-6AV UV-Vis detector, two LC-6A pumps, and a C-R3A integrator). The solvent program used a linear gradient of 5 to 80% B over 10 min, 80% B for 5 min, then 80 to 5% B over 5 min (solution A, 10 mM potassium phosphate buffer, pH 6.0; solution B, methanol). Reactions (1 mL) were run in 50 mM potassium phosphate (pH 7.4) containing 200 μM NADH, 50 μM SN, and HT-29 or BE cytosol. NADH oxidation was quantified at 340 nm following 30-min incubations at 22°. Reactions were also run in the presence of a selective DTD inhibitor, dicumarol (20 μM).

Oxygen Uptake

Oxygen uptake experiments were performed with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH) using air-saturated water for calibration. The oxygen content of air-saturated water was adjusted for temperature and altitude. Reactions (3 mL) were run in 50 mM potassium phosphate (pH 7.4) containing 200 μM NADH, 50 μM SN, and HT-29 or BE cytosol. Oxygen uptake was followed over a 30-min period at 22°. Reactions were also run in the presence of dicumarol (20 μM) and an iron chelator, deferoxamine (100 μM).

Drug Treatment

Drug treatments were performed as previously described [26]. Cells were exposed to SN for 2 hr.

Cytotoxicity Assay

Inhibition of colony-forming ability in HT-29 and BE cells was determined using a standard clonogenic assay as previously described [26].

Genotoxicity Assay

Genotoxicity in the HT-29 and BE cells was assessed using an alkaline elution method for determining the extent of DNA single-strand breaks. Alkaline elution experiments were carried out as described [37] and modified [26]. In addition, experiments were performed to study the capacity for repair of DNA strand breaks in these cells. After a 2-hr drug treatment, the drug solution was removed and replaced with drug-free medium. The cells were incubated for 0, 6, 12 and 24 hr to allow the cells to repair their DNA. Alkaline elution experiments were then performed as above.

Bioreductive Enzyme Activities in HT-29 and BE Cells

DTD activity was measured as the dicumarol-sensitive reduction of DCPIP as described [26]. P450R activity was measured as NADPH-dependent reduction of cytochrome c according to

the method of Vermillion and Coon [38], and B5R activity was measured as the NADH-dependent reduction of cytochrome c. XO and XDH activities were determined by following the formation of uric acid from xanthine by the method of Stirpe and Della Corte [39]. Enzymes were assayed using cell cytosol with the exception of P450R and B5R, which were measured using cell sonicates.

RESULTS

Reduction of SN by HT-29 and BE cytosol was quantified by following the oxidation of NADH in the presence and absence of dicumarol, a selective inhibitor of DTD. These results are presented in Fig. 1. Dicumarol-sensitive oxidation of NADH was observed with HT-29 cytosol, but not with BE cytosol, suggesting that DTD was responsible for the reduction of SN by HT-29 cytosol.

Since the DNA-damaging effects of SN are dependent on its ability to redox cycle [16], oxygen uptake by HT-29 and BE cytosol was also investigated (Table 1). Consistent with the HPLC data, reduction of SN by HT-29 cytosol in the presence of NADH led to substantial oxygen uptake while no oxygen uptake was observed with BE cytosol. HT-29 cytosol-mediated oxygen uptake was inhibited by 20 μM dicumarol, which again suggested that DTD was responsible for the activation of SN by HT-29 cytosol. Oxygen uptake by HT-29 cytosol was not inhibited by 100 μM deferoxamine, an iron chelator.

The cytotoxicity of SN was studied in the HT-29 and BE cells using a clonogenic assay, and the results are presented in Fig. 2. SN was much more toxic to the DTD-rich HT-29 cells than to the DTD-deficient BE cells. A concentration of 40 nM SN produced only about 1% survival in HT-29 cells, whereas in BE cells 400 nM led to greater than 10% survival, suggesting that SN was selectively toxic to the high DTD cell line. In

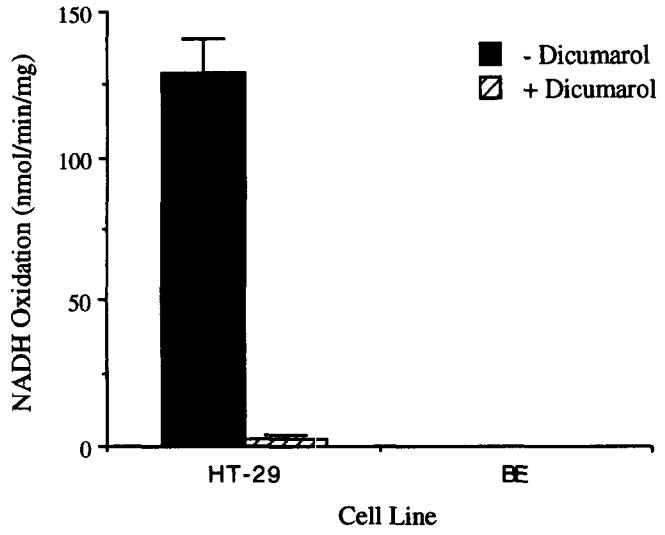

FIG. 1. Metabolism of SN by HT-29 and BE cytosol as measured by NADH oxidation. Reactions contained 200 μM NADH, 50 μM SN, and HT-29 or BE cytosol in 50 mM potassium phosphate buffer (pH 7.4). Values are means ± SD for N = 3 experiments.

TABLE 1. Oxygen consumption from SN metabolism by HT-29 and BE cytosol

Cell line	Oxygen consumption (nmol · min^{-1} · mg^{-1})
HT-29	
Cytosol only	93.9 ± 12.9*
+100 μM Deferoxamine	97.3 ± 4.7
+20 μM Dicumarol	ND†
BE	
Cytosol only	ND

Reactions contained 200 μM NADH, 50 μM SN, HT-29 or BE cytosol, and 50 mM potassium phosphate buffer (pH 7.4) at 22°. Control rates were <0.5 nmol/min.
*Mean ± SD for N = 3 experiments.
† ND, <detectable.

Fig. 3, the effects of the iron chelator deferoxamine on toxicity are shown. Deferoxamine decreased toxicity in both HT-29 and BE cells. Since production of hydroxyl radicals in a Fenton-type reaction requires a metal-ion catalyst, this provides evidence for the role of hydroxyl radicals in the toxicity of SN. In control experiments, deferoxamine had no effect on cell survival at the concentrations used.

Genotoxicity was assessed in the HT-29 (Fig. 4) and BE (Fig. 5) cells using alkaline elution. The HT-29 and BE cells were labeled with [^{3}H]thymidine, and L1210 cells labeled with [^{14}C]thymidine were used as an internal control. All cells were irradiated, which causes DNA fragmentation. Larger molecular weight fragments are retained on polycarbonate filters so a decrease in the amount of [^{3}H]DNA retained is indicative of DNA strand breaks. As illustrated in Figs. 4 and 5, increasing SN concentration resulted in a higher incidence of DNA strand breaks. As expected, this effect was greater for the high

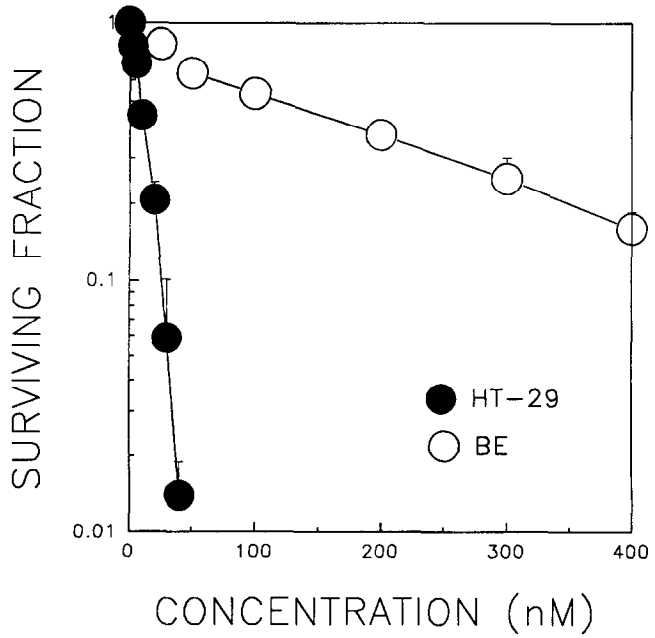

FIG. 2. Cytotoxicity of SN to HT-29 and BE cells via clonogenic assay. Values are means ± SD for a minimum of N = 3 experiments.

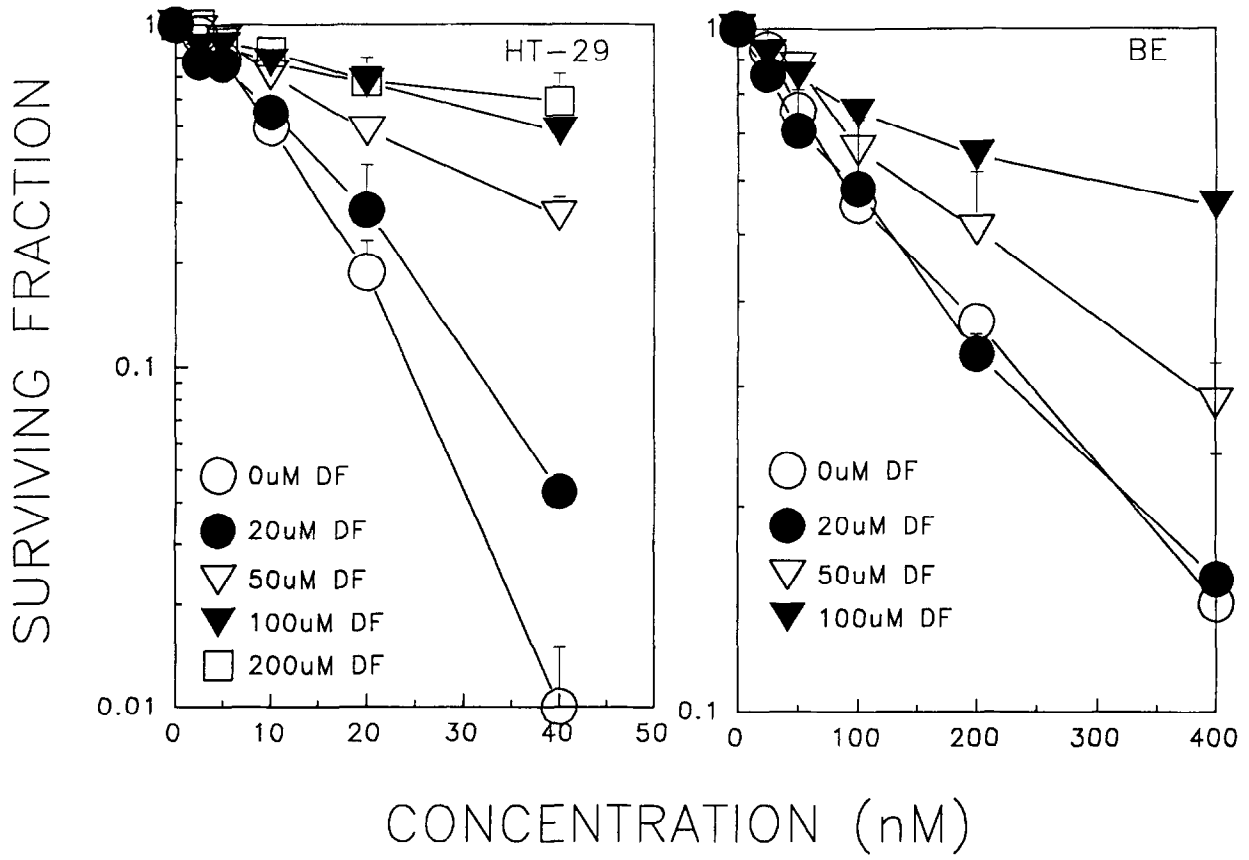

FIG. 3. Cytotoxicity of SN to HT-29 and BE cells in the presence and absence of deferoxamine (DF). Values are means ± SD for a minimum of N = 3 experiments.

DTD HT-29 cells than for the BE cells, which had no detectable DTD activity. The protective effect of deferoxamine that was observed in the cytotoxicity studies was also seen in the genotoxicity studies (Figs. 4 and 5). Deferoxamine protected against DNA strand breaks in both HT-29 and BE cells, suggesting that hydroxyl radicals produced by the redox cycling of SN were responsible for the DNA strand breaks. Some DNA repair was also evident in both cell lines, since a decrease in the amount of strand breaks was observed when the drug was removed and cells were incubated for an additional 6, 12 and 24 hr in drug-free medium prior to alkaline elution analysis.

DISCUSSION

SN is a redox-cycling antitumor quinone that is believed to cause DNA damage by producing hydroxyl radicals [16, 20]. Selective activation of SN in tumor cells with elevated levels of DTD could provide a means of increasing antitumor efficacy and decreasing toxicity to non-cancerous cells. Recently, we showed that SN was an excellent substrate for both rat and human recombinant DTD, that reduction of SN by DTD led to stoichiometric oxygen consumption, and that SN was selectively toxic to an NSCLC cell line (H460) with high DTD activity versus an NSCLC cell line (H596) with undetectable DTD activity [31]. In fact, SN was 86-fold more toxic to the H460 cell line than to the H596 cell line based on IC_{50} values. When studied in the NCI human tumor cell line panel, SN showed the best correlation between DTD activity and toxicity of the over 31,000 compounds tested [40].

In the present study, we have compared SN metabolism, genotoxicity, and cytotoxicity in two human colon cancer cell lines: HT-29 with high DTD activity and BE with undetectable DTD activity. The BE cell line has a point mutation in the DTD gene which prevents the expression of an active DTD protein [41]. The bioreductive enzyme profiles for these two cell lines are presented in Table 2. While there are minor differences in some of the other bioreductive enzymes in the two cell lines, the difference in DTD activity is clearly the most significant. These results are consistent with those reported earlier [26].

SN metabolism was observed in HT-29 cytosol in the presence of NADH, and metabolism was inhibited by dicumarol. Conversely, no metabolism was detected using BE cytosol. Similar results were seen in the oxygen uptake studies. With HT-29 cytosol, nearly stoichiometric oxygen uptake was observed, which was inhibited completely by 20 μM dicumarol. As expected, BE cytosol produced no detectable oxygen uptake. These results suggested that DTD played an important role in the bioactivation of SN in HT-29 cells and that SN could be activated selectively in tumor cells with elevated DTD activity. Interestingly, deferoxamine had no effect on the auto-oxidation of the SN hydroquinone following reduction

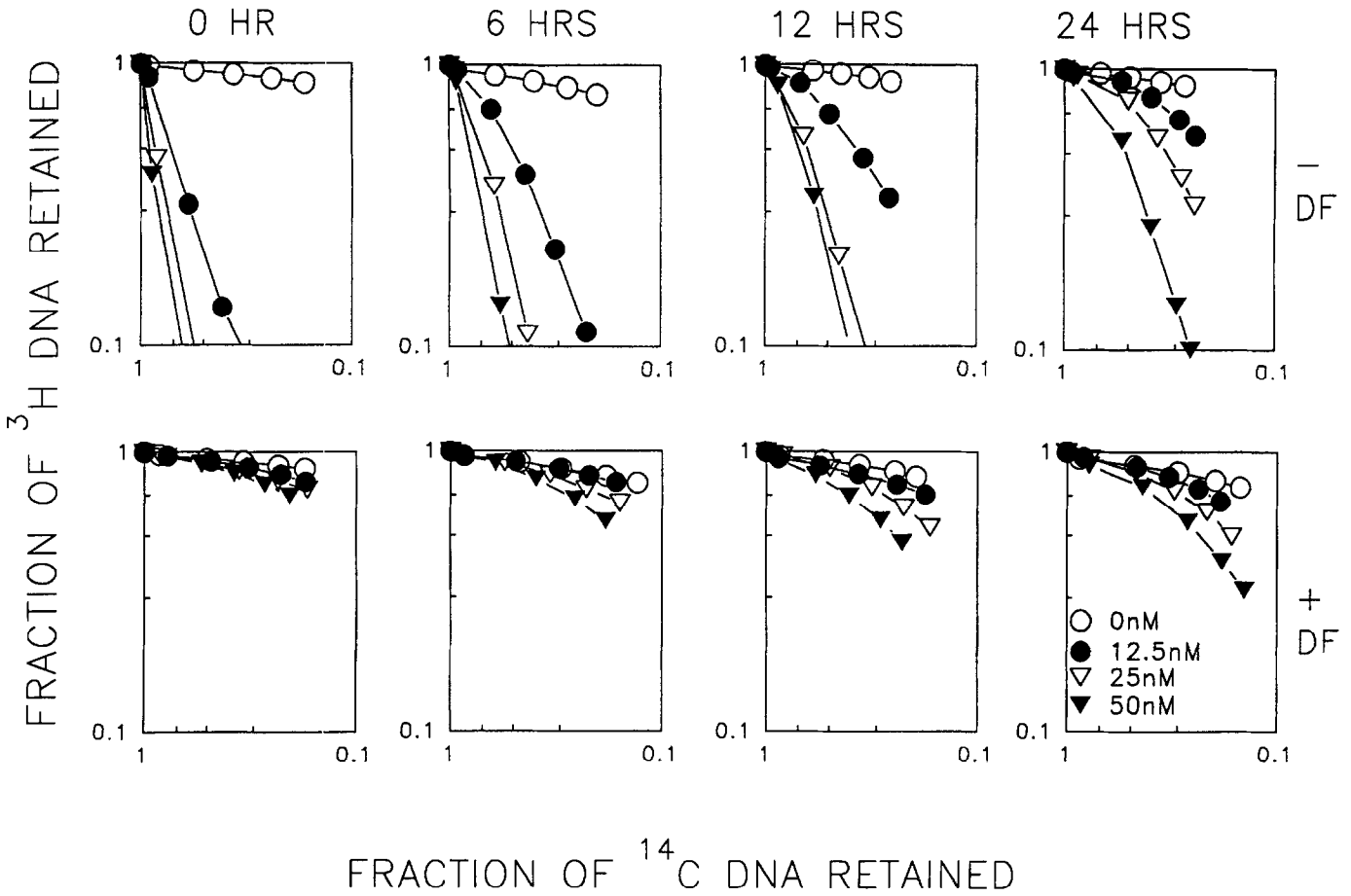

FIG. 4. Genotoxicity of SN (0–50 nM) in HT-29 cells in the presence and absence of 100 μM deferoxamine (DF). Alkaline elution was carried out at 0, 6, 12, and 24 hr after removal of SN to assess DNA repair capacity. SN concentrations: 0 nM (○), 12.5 nM (●), 25 nM (▽), 50 nM (▼).

by DTD since oxygen uptake was unchanged essentially in the presence of deferoxamine in the HT-29 studies. This was somewhat surprising, since Lown and coworkers [16] reported that complexes of divalent metal cations with reduced quinolinequinone model compounds were important for the autoxidation of those hydroquinones. Our results suggest that such complexes are not necessary for efficient redox cycling of SN reduced by DTD.

In cytotoxicity studies, SN was found to be much more toxic to the high DTD HT-29 cells than to the DTD-deficient BE cells. As described above, this was consistent with our earlier work with lung tumor cell lines. These results further support the role of DTD as a potential target enzyme for cytotoxic drugs that are good substrates for DTD, such as SN. Toxicity was decreased by increasing concentrations of deferoxamine in both cell lines, but the effect was greatest with the HT-29 cell line. This suggests that hydroxyl radicals ($OH^{\bullet}$) produced by redox cycling of SN may be responsible for the cytotoxicity observed with SN (Fig. 6). Superoxide ($O_2^{\bullet-}$) anions are generated from the interaction of oxygen and the SN hydroquinone. H_2O_2 is formed from the dismutation of superoxide, and in the presence of reduced iron (Fe^{2+}), the highly reactive hydroxyl radicals ($OH^{\bullet}$) may be produced via a Fenton reaction. The oxidized iron (Fe^{3+}) that results can be reduced to Fe^{2+} by additional superoxide molecules, and the process may be repeated. Deferoxamine blocks this reaction by chelating divalent iron, and in the present study, toxicity was decreased by deferoxamine, supporting the role of hydroxyl radicals ($OH^{\bullet}$) in the toxicity of SN. In the BE cell line, one-electron reductases may generate SN semiquinone radicals that, after reaction with oxygen, may produce $O_2^{\bullet-}$ and H_2O_2. Although NADH-dependent redox cycling was not observed in BE cytosol, some of the more important one-electron reductases are known to be NADPH dependent, such as P450R. Low levels of P450R have been detected in BE cells [26], and this may explain the observation that, although SN is markedly less toxic to BE cells, toxicity can be inhibited with deferoxamine.

A much higher incidence of DNA strand breaks was observed in the HT-29 cells than in the BE cells upon exposure to SN. Genotoxicity from DNA strand breaks was probably responsible for the increased cytotoxicity that was also observed in the HT-29 cells. Again, this adds to the evidence supporting the role of DTD in the bioactivation of SN in the DTD-rich HT-29 cell line. Deferoxamine was effective in preventing strand breaks in both HT-29 and BE cells, indicating that hydroxyl radicals may be responsible for the DNA strand breaks produced in these cells. Some DNA repair was observed since the amount of DNA damage was reduced when cells

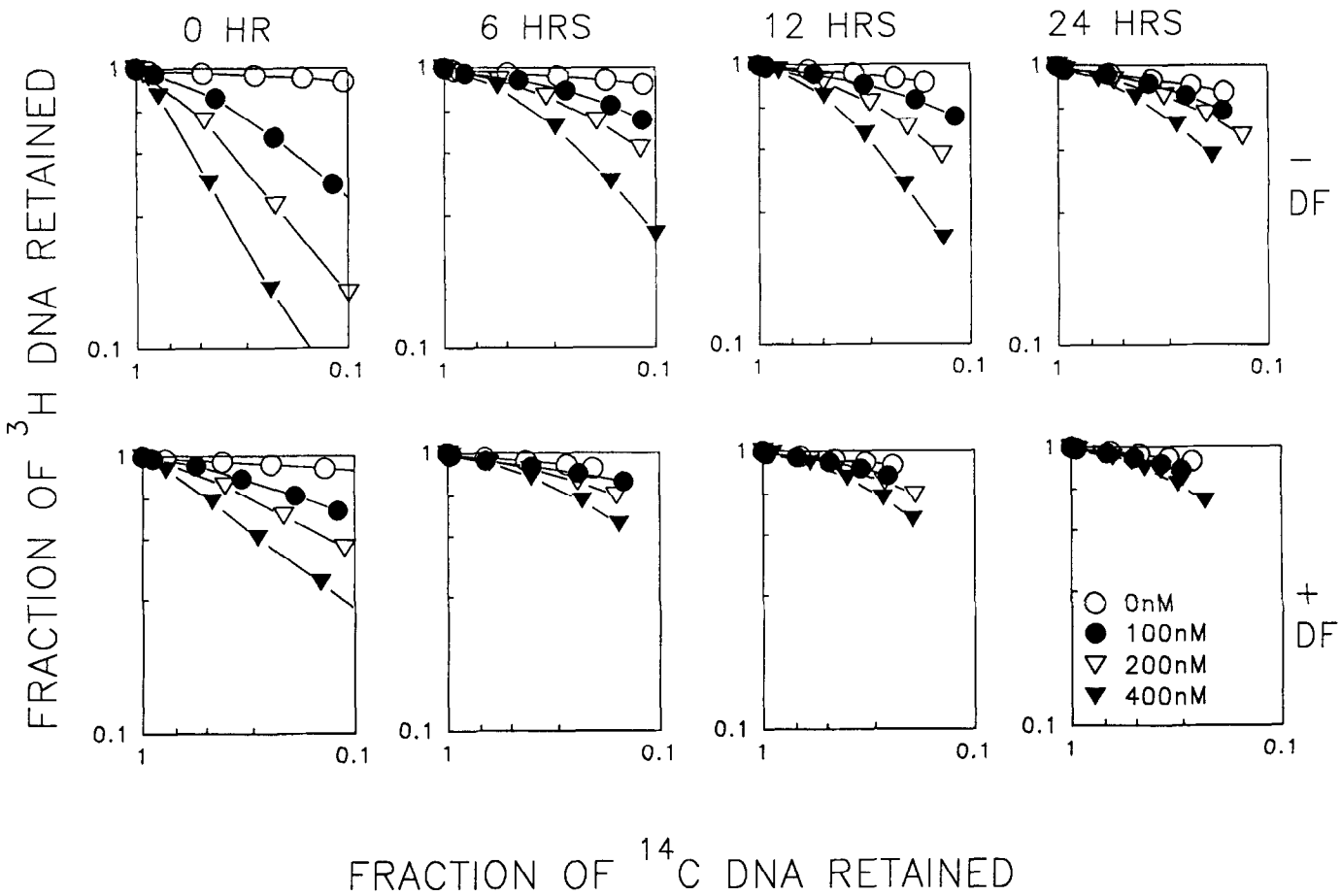

FIG. 5. Genotoxicity of SN (0–400 nM) in BE cells in the presence and absence of 100 μM deferoxamine (DF). Alkaline elution was carried out at 0, 6, 12, and 24 hr after removal of SN to assess DNA repair capacity. SN concentrations: 0 nM (○), 100 nM (●), 200 nM (▽), and 400 nM (▼).

were incubated for up to 24 hr following SN exposure prior to alkaline elution. The ability of cells to repair DNA damage in the form of DNA strand breaks could be an important consideration when determining dosage and frequency of administration of potential cytotoxic drugs such as SN.

In this report, metabolism, cytotoxicity, and genotoxicity of SN in DTD-rich HT-29 and DTD-deficient BE human colon carcinoma cells were evaluated. Reduction and oxygen consumption were observed in HT-29 cytosol, but were not detectable in BE cytosol. Cytotoxicity and genotoxicity were also much greater in HT-29 cells than in BE cells. In addition, evidence was presented for the role of hydroxyl radicals in both cytotoxicity and genotoxicity in these cells. These data establish the importance of DTD in the bioactivation of SN in a high DTD cell line, and suggest that SN, if its myeloid toxicity [5–7] can be controlled, may be an effective agent for the treatment of tumors with elevated DTD activity.

TABLE 2. Bioreductive enzyme activities in HT-29 and BE cell lines

Enzyme	Enzyme activity (nmol · min^{-1} · mg^{-1})	
	HT-29 cells	BE cells
DTD	1240 ± 230*	ND†
P450R	5.42 ± 0.13	6.57 ± 0.16
B5R	6.62 ± 0.06	6.35 ± 0.11
XO	ND	ND
XDH	ND	ND

* Mean ± SD for N = 3 experiments.
† ND, <detectable.

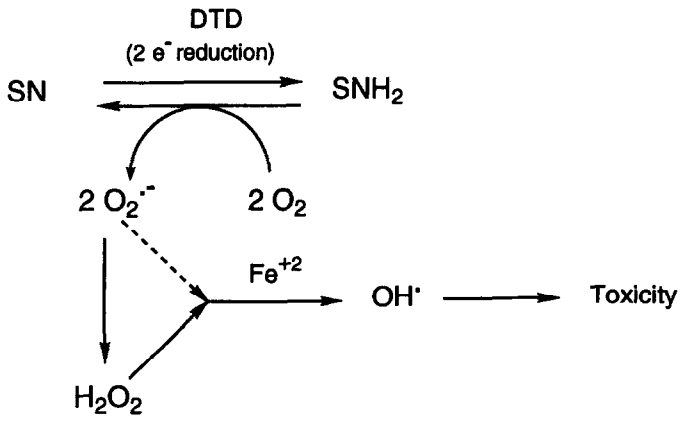

FIG. 6. Bioactivation of SN by DTD to cytotoxic species.

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