O_2-Dependent Cleavage of DNA by Tetrazomine†

Robert M. Williams,* Mark E. Flanagan, and Tracy N. Tippie

Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

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ABSTRACT: A mechanism for the reduction of molecular oxygen that results in the O_2-dependent cleavage of both single-stranded and double-stranded DNA by the antitumor antibiotic tetrazomine (**1**) is presented. The results are discussed in the context of a redox self-disproportionation of the oxazolidine moiety of tetrazomine. Comparisons are made to the structurally analogous natural product quinocarcin (**2**) in which, like tetrazomine, the oxazolidine moiety is invoked in redox chemistry, which ultimately results in the reduction of molecular oxygen to superoxide.

It is now widely recognized (Fisher & Aristoff, 1988; Remers, 1985) that a variety of clinically significant antitumor antibiotics can mediate oxygen-dependent cleavage of the ribose phosphate backbone of cellular DNA and RNA. An enormous structural array of interesting natural products and semisynthetic and totally synthetic substances mediates oxidative strand scission of nucleic acids through three main families of reactions: (1) metal-mediated activation of O_2, ultimately producing hydroxyl radical or other reactive oxygen species (Stubbe & Kozarich, 1987; Walling, 1975); (2) non-metal-dependent generation of reactive carbon radicals (Myers, 1987; Nicolaou et al., 1988; Magnus et al., 1988; Hawley et al., 1989) that mediate CH abstraction from the deoxyribose backbone (the resulting deoxyribosyl radical subsequently reacts with molecular oxygen, culminating in strand scission); and (3) photolytic production of hydroxyl radical (Saito et al., 1990; Zafirou & Bonneau, 1987), which does not require metal participation for the DNA cleavage event. A rich array of chemistry can be found in the metal-dependent family of DNA-damaging agents. Many readily oxidizable organic substances are capable of reducing molecular oxygen, resulting in the production of superoxide, such as semiquinone radical anions, thiols (Misra, 1974), and ascorbate, among others. Superoxide is well-documented (Lesko & Lorentzen, 1980) to mediate DNA strand breakage via dismutation to hydrogen peroxide followed by Fenton chemistry [in the presence of Fe(III)], generating the highly reactive hydroxyl radical.

Tetrazomine (**1**) is a natural secondary metabolite that was recently isolated from *Saccharothrix mutabilis* subsp. *chichijimaensisi* by Yamanouchi Pharmaceutical Co. (Japan) (Suzuki et al., 1991) and is the most recent member of the quinocarcin (**2**)/naphthyridinomycin/saframycin class of antitumor agents. Tetrazomine has been shown to display broad antimicrobial activity against both Gram-positive and Gram-negative bacteria *in vitro*. Tetrazomine also displays promising *in vitro* antitumor activity (Suzuki et al., 1991) against lymphoid leukemia L1210 and P388 leukemia at 0.0427 and 0.014 μg/mL, respectively. This substance also displayed antitumor activity against P388 leukemia *in vivo*.

Our interest in this substance stems from structural similarities between tetrazomine and the antitumor antibiotic quinocarcin (**2**), which has been the subject of considerable investigation (Williams et al., 1991, 1992). Like quinocarcin, tetrazomine (1) is capable of cleaving both synthetic oligonucleotides and plasmid DNA in an O_2-dependent manner in the absence of external reducing agents such as dithiothreitol (DTT); (2) is not stimulated by the addition of metal ions [Fe(II), Fe(III)]; (3) is inhibited by free radical scavengers such as picolinic acid (Sheu et al., 1990); and (4) is inhibited by superoxide dismutase (SOD[1]) and catalase. We have recently obtained experimental evidence, presented herein, that tetrazomine undergoes a redox self-disproportionation reaction similar to that observed for quinocarcin, which may be coupled to the capacity of this substance to affect the production of superoxide in the presence of molecular oxygen and results, at least in part, in Fenton-mediated lesions in DNA; a proposed mechanism for this process is presented in Figure 2. The rate at which tetrazomine produces superoxide has been carefully examined by following the reduction of nitroblue tetrazolium (NBT) spectrophotometrically under various conditions. It was found that, in addition to being completely inhibited by SOD, these experiments closely paralleled trends observed for the nicking of supercoiled plasmid DNA by tetrazomine.

MATERIALS AND METHODS

Disproportionation of Tetrazomine and Analysis of Products. A 10 mM solution of **1** was prepared in 20 mM phosphate buffer (pH 8.0), which was then deoxygenated by freeze–thaw purging with nitrogen. A sample was then analyzed by HPLC (resolve pack C18 column; 10 mM sodium sulfate, 3% acetonitrile, 0.05% acetic acid, and 0.002% 1-pentanesulfonate sodium salt (isocratic); detected by UV at 254 nm), which revealed only a peak at 5 min corresponding to **1**. The solution was then aged at 4 °C under anaerobic conditions, with aliquots being taken periodically (every third day for 2 weeks), and analyzed by HPLC for tetrazomine and the formation of new products. Approximately 20% conversion of tetrazomine to new products was observed after 2 weeks. These breakdown products were isolated (by HPLC) and desalted by trituration with methanol. Following filtration, the methanol solutions were concentrated under reduced pressure, redissolved in ddH_2O, passed through a column of Dowex 1 (Cl^- form), and lyophilized. One product was identified as the reduced form of tetrazomine, which henceforth will be referred to as tetrazominol (**4**). Authentic tetrazominol was obtained from

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: SOD, superoxide dismutase; NBT, nitroblue tetrazolium; TE, 100 mM Tris base/10 mM EDTA; EDTA, ethylenediaminetetraacetic acid; ddH_2O, double deionized water.

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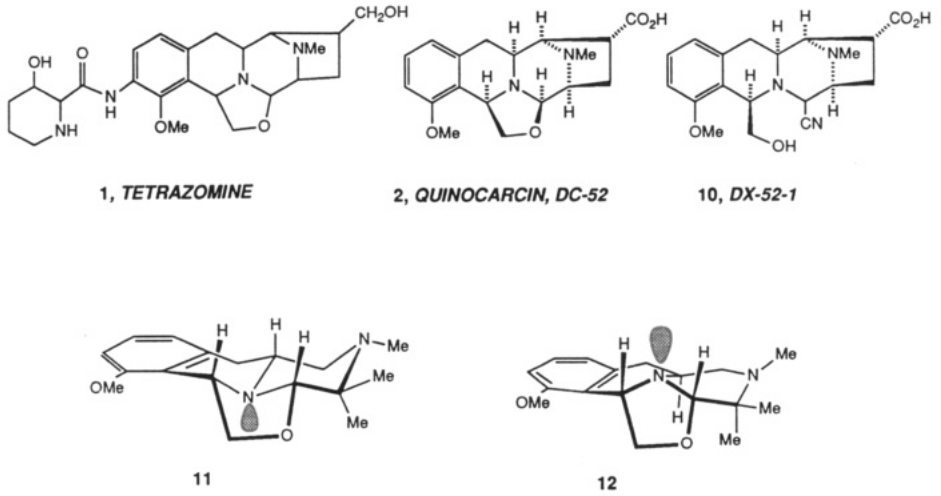

FIGURE 1: Tetrazomine and structural analogs.

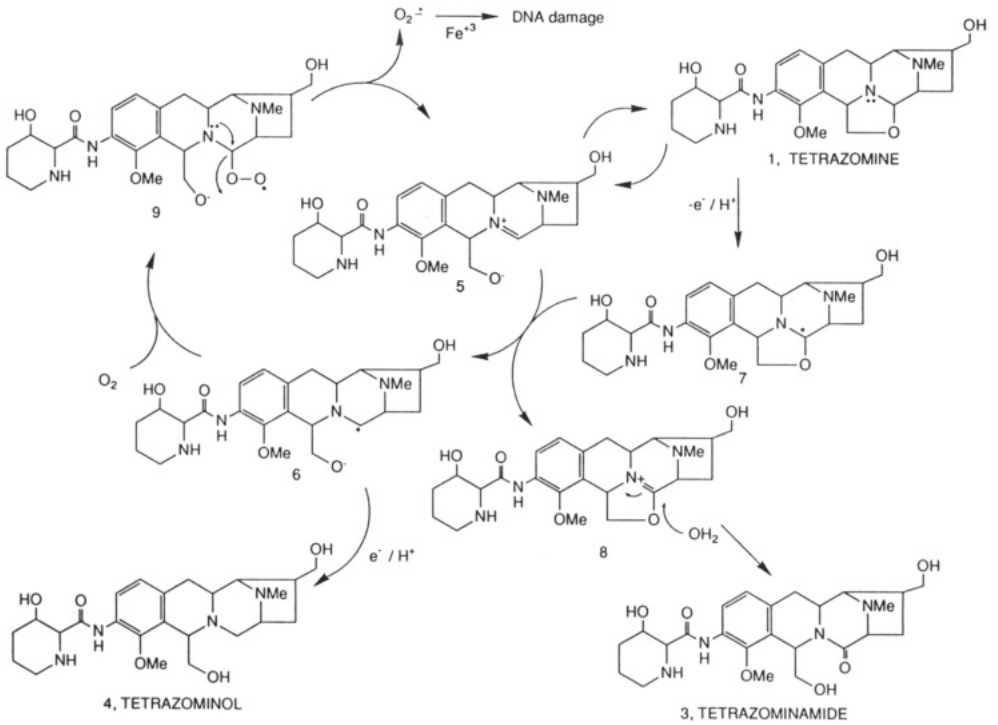

FIGURE 2: Proposed disproportionation of tetrazomine: under anaerobic conditions, resulting in the formation of **3** and **4**; under aerobic conditions, resulting in the production of superoxide.

tetrazomine by reacting 10 mg of the pure drug with 10 equiv of $NaBH_4$ (Baker) in 3 mL of methanol (0 °C) for 3 h. The sample of tetrazominol obtained following isolation by preparative thin-layer chromatography (silica; 4:1 CH_2Cl_2/methanol, $R_f = 0.2$) had the exact same HPLC retention time (8 min) and molecular weight by mass spectrometry as **4** collected from the disproportionation of **1** described above: ^{1}H NMR (300 MHz) (D_2O) δ (HOD) 1.63–1.94 (5H, m), 2.24 (1H, t, J = 11.4 Hz), 2.48 (1H, d, J = 14.1 Hz), 2.57 (1H, q, J = 11.5 Hz), 2.79–2.89 (5H, m), 2.96 (1H, d, J = 16.3 Hz), 3.00 (1H, d, J = 12.9 Hz), 3.18 (1H, br s), 3.36 (1H, d, J = 13.8 Hz), 3.53–3.68 (5H, m), 3.60 (3H, s), 3.84–3.90 (2H, m), 4.17 (1H, d, J = 1.8 Hz), 4.56 (1H, s), 6.89 (1H, d, J = 8.2 Hz), 7.33 (1H, d, J = 8.2 Hz). MS m/e (M^+): 460.26960 (calcd for $C_{24}H_{36}N_4O_5$, 460.2696). Another product isolated from the disproportionation by HPLC (retention time, 4 min) was identified as the oxidized form of tetrazomine, which henceforth will be referred to as tetrazominamide (**3**): MS m/e (M^+) 475.2578 (calcd for $C_{24}H_{35}N_4O_6$, 475.258). The other material formed was an unidentifiable decomposition product. We were not able to obtain a sufficient quantity of tetrazomine from Yamonouchi Co. to rigorously isolate and characterize this material.

Reductions of Nitroblue Tetrazolium by Tetrazomine. Each reaction was performed in triplicate by adding an appropriate amount of tetrazomine stock solution (20 mM) to an aerated solution of nitroblue tetrazolium (0.12 mM) in 20 mM phosphate buffer (at the indicated pH) containing 1% Triton X-100 detergent with the final volume brought to 750 μL with ddH_2O. The optical absorbance was measured at 25 °C over a 30-min period at 500 nm (Varian DMS 80 UV/vis spectrophotometer), and the ΔOD was the average slope for the linear OD change over the reaction time. The rates for superoxide production were calculated by assuming that $[O_2]$ does not appreciably change over this time period and is in excess (zero order in oxygen). The rates (reported in Table 1) were calculated from the ΔOD measurements and based

Table 1: Rates of Superoxide Production: Measurements by Reduction of Nitroblue Tetrazolium (NBT)

entry	substrate	pH	rate ($\mu s^{-1} \times 10^{-9}$)
1	1.0 mM tetrazomine	6	2.46
2	1.0 mM tetrazomine	7	10.6
3	1.0 mM tetrazomine	8	17.5
4	1.0 mM quinocarcin	8	1.1
5	1.0 mM tetrazomine + 10 μg/mL SOD	8	0.0
6	1.0 mM DX-52-1	8	0.0
7	1.0 mM **11**	8	0.41
8	1.0 mM **12**	8	0.0
9	20 mM phosphate buffer (control)	8	0.0

on a molar extinction coefficient (ϵ_0) of 12 200 for the formazan product of NBT at 500 nm.

Preparation and Purification of Supercoiled Plasmid DNA (pUC 19). To 40 μL of electrocompetent cells (*E. coli*, MC 1061) was added 1 μL (0.35 μg) of pUC 19 plasmid DNA (New England Biolabs), and the mixture was agitated and aged in an ice bath for 1 min. The mixture was then transferred to an ice-cooled, 0.1-cm electroporation cuvette, and the material was pulsed (Bio-Rad gene pulser) at 25 μF, 200 Ω, and 1.1 kV. The cells were then quickly transferred to 1 mL of sterile SOC broth (20 mM glucose, 20 g of bacto-tryptone (Difco), 5 g of bacto-yeast (Difco), 1 g of $MgCl_2$, 0.5 g of NaCl, and 186 mg KCl to 1.0 L) and incubated at 37 °C, with agitation (200 rpm), for 1 h. The mixture was transferred to a sterile Eppendorf tube and centrifuged (14 000 rpm, 5 min, room temperature). The supernatant was discarded, and the cells were resuspended in 400 μL of sterile SOC broth. Dilutions were made to 10^2, 10^4, 10^6, 10^8 (400 μL each), and 100 μL of each plated onto LB agar plates containing 30 μg/mL ampicillin (in duplicate). The plates were inverted and incubated at 37 °C for 18 h, at which time colonies were counted and a yield of 10^{10} transformants was assessed. A resistant colony was then grown in 500 mL of sterile LB broth (10 g of bacto-tryptone, 5 g of bacto-yeast extract, and 10 g of NaCl to 1.0 L) containing 30 μg/mL ampicillin at 37 °C for 18 h. The cells were pelleted by centrifugation (5000 rpm, 10 min, 4 °C). Plasmid DNA (pUC 19) was then isolated following the Promega Magic Maxi-prep kit procedure. The supercoiled plasmid was further purified by low-melt agarose gel electrophoresis (1.2%). The band corresponding to supercoiled plasmid (visualized by 0.4 μg/mL ethidium bromide using a hand-held UV light) was cut from the gel and melted at 75 °C. To this was added 4 vol of 37 °C TE, and the mixture was aged at 37 °C for 1 h. To this solution was then added 1 vol of Tris-equilibrated phenol (pH 8) (room temperature), the tube was mixed gently by rocking, and the layers were separated by centrifugation (4000 rpm, 15 min, room temperature). The aqueous layer (top) was removed and extracted with 2 × 2 vol of butanol (centrifugation was necessary for the separation of layers). To the aqueous layer (bottom) were added 3 vol of ethanol and 50 μL of 3 M NaOAc, pH 5.2, and the solution was mixed gently, aged at −70 °C for 10 min, and centrifuged (20 000 rpm, 30 min, 4 °C). The supernatant was discarded and the pellet resuspended in 200 μL of sterile deionized water. Supercoiled plasmid DNA (pU 19) was recovered in 90% yield from the low-melt gel.

Cleavage of Supercoiled Plasmid DNA (pUC 19). DNA nicking reaction mixtures were made up by the addition at 0 °C of appropriate amounts of reagent stock solutions to a stock solution of supercoiled plasmid DNA (pUC 19), prepared as described above and containing 0.15 μg of DNA per reaction (23 μM base-pair concentration). The total volumes of the reaction mixtures were brought to 10 μL each with distilled, deionized water when necessary, and the reaction mixtures were incubated at 37 °C for 2 h in tightly capped plastic Eppendorf tubes. Stock solutions for experiments including DNA were prepared using distilled, deionized water and commercially available reagents: sodium phosphate, monobasic, EM Science; sodium phosphate, dibasic and 30% hydrogen peroxide, Malinckrodt; superoxide dismutase and beef liver catalase (suspension in water), Boehringer Mannheim Biochemical. Desferal was the generous gift of Ciba-Geigy Co. The cleavage of plasmid DNA was detected by loading the reactions onto 1.2% agarose gels containing 0.4 μg/mL ethidium bromide and running for 2 h at 55 V. The electrophoresis gels were immediately visualized on a UV transilluminator and photographed using black and white instant films (Polaroid T667). The measurements of the relative intensities of DNA bands were performed on the photographs using a Dell System 325 computer and Technology Resources Inc. image processing software. The film used to photograph the gels was confirmed to have a linear response to the range of DNA quantities used. The mean number of single-strand scissions (S) per supercoiled DNA substrate was calculated using the Poisson distribution (Hertzberg & Dervan, 1984), where the value S for the DNA control represents the amount of nicked open circular DNA present in the starting plasmid, and was subtracted from the S values calculated for the individual cleavage reactions. When only forms I (supercoiled) and II (nicked open circular) are present, the equation simplifies to $S = -\ln f_I$, where f_I is the fraction of form I molecules from the densitometry data. In those cases where form III (linear) DNA is present, S is calculated from $f_I + f_{II} = [1 - S(2h + 1)/2L]^{S/2}$, where h is the distance between hits on opposite strands to produce a linear molecule (16 base pairs) and L is the total number of base pairs in pUC 19 (2686 base pairs).

Labeling (5′-^{32}P) and Reactions of Synthetic 45-mer for High-Resolution Polyacrylamide Gel Electrophoresis. To a solution of the synthetic deoxyoligonucleotide (100 pmol) in 74 μL of ddH_2O were added 20 μL of polynucleotide kinase buffer, 4 μL (40 units) of T4 polynucleotide kinase (New England Biolabs), and 1 μL (10 μCi) of [γ-^{32}P]ATP (DuPont). The reaction was incubated for 60 min at 37 °C and for 10 min at 70 °C. The solution was loaded onto a 2-mL column of Sephadex G-50 and eluted with TE buffer. The first radioactive fraction was collected and precipitated with ethanol/3 M NaOAc (pH 5.2) and dried. Annealing was performed by mixing equimolar amounts of the 5′-^{32}P-end-labeled strand and the complementary strand in ddH_2O to a final concentration of 2 pmol/μL; each was heated to 65 °C for 30 min and slowly cooled to 0 °C. Reactions were made up by the additions at 0 °C of appropriate amounts of stock solutions to 3 μL each of labeled DNA (5 pmol). The total volumes of the reactions were brought to 16 μL each with ddH_2O and enough 80 mM phosphate buffer (pH 8) so as to achieve final concentrations of 20 mM phosphate. Each reaction was incubated at 37 °C for 5 h (none of the reactions contained any additional reducing agents, such as DTT). To the reaction mixtures were then added 1 μL of 3 M NaOAc (pH 5.2) and 60 μL of ethanol, and the resulting solutions were aged at −70 °C for 10 min. Each tube was centrifuged at 14 000 rpm for 10 min at 4 °C, the supernatants were discarded, and the DNA pellets were dried under reduced pressure. To each dried pellet of DNA was added 10 μL of loading buffer (formamide, 10 mM EDTA (pH 8), 0.025%

xylenecyanol FF, and 0.025% bromophenol blue), and the sample was then heated to 90 °C for 5 min, placed on ice, and immediately loaded (20 000 cpm/lane as measured using a Packard 1500 liquid scintillation counter) onto a 20% denaturing (urea) polyacrylamide gel and run for 6 h at 1300 V. The bands were visualized by autoradiography.

RESULTS

Disproportionation of Tetrazomine. Natural tetrazomine, obtained from Yamanouchi Pharmaceutical Co., Ltd., was allowed to stand in carefully deoxygenated phosphate buffer (20 mM, pH 8) at 4 °C, with new products being produced. The identification of the anaerobic redox products **3** and **4** supports the proposal that tetrazomine serves as its own reductant, since no other reducing agents were present. Therefore, on the basis of the analogous chemical structure, the observation of anaerobic disproportionation products, and the analogous aerobic behavior (i.e., superoxide production) that tetrazomine shares with quinocarcin, we propose the same Cannizzaro-driven reduction of molecular oxygen for tetrazomine. Therefore, as suggested in Figure 2, single-electron transfer from **1** with concomitant proton loss from the oxazolidine nitrogen to the ring-opened tautomer **5** would furnish radical anion **6** and the oxazolidinyl radical **7**. Radical **7** should be capable of reducing a second equivalent of **5**, ultimately becoming oxazolidinium ion **8**, which would hydrolyze to tetrazominamide (**3**). Under anaerobic conditions, radical anion **6** subsequently suffers a second electron transfer (presumably from **1** or **7**) with concomitant protonation, resulting in tetrazominol (**4**). Under aerobic conditions, radical anion **6** can react with molecular oxygen to produce peroxy radical anion **9** which, with nitrogen participation, expels 1 molar equiv of superoxide, regenerating **5**.

Reductions of Nitroblue Tetrazolium by Tetrazomine. The rate of superoxide production was carefully followed by reduction of NBT (Misra, 1974; Tsou, 1956) under various pH conditions, and it was found that these reactions are completely inhibited by SOD, thus supporting the role of superoxide in this mechanism; the results are collected in Table 1. The reduction of NBT by tetrazomine is pH-dependent, exhibiting an increased rate of reduction as the pH is raised (Table 1, entries 1–3). This behavior directly parallels the pH dependency for the plasmid DNA cleavage reactions described below. Entries 6–8 of Table 1 support the notion that the oxazolidine moiety of tetrazomine must be "intact" and in the quinocarcin configuration, exhibited by synthetic analog **11**, in order for the production of superoxide to proceed.

Cleavage of Supercoiled Plasmid DNA (pUC 19) by Tetrazomine. In order to examine the interaction of tetrazomine with DNA, the pure antibiotic (0.01–10 mM) was allowed to react with supercoiled plasmid DNA (pUC 19) in phosphate buffer (20 mM) between pH 5 and 9 at 37 °C for 2 h in the presence of air. Salient experimental results are collected in Tables 2 and 3. Tetrazomine displayed nicking of the DNA at a 0.1 mM concentration (Table 2, entry 8) at pH 8 without the addition of any external reductants. The reaction was found to be pH-dependent; optimal cleavage occurred at or above pH 7. At lower pH values (below pH 6), nicking was observed, but to a lesser extent (Table 2, entries 1–5). This is consistent with the obligate participation of the *unprotonated* oxazolidine nitrogen atom in the redox cycle. The slightly different pH versus nicking yield (S) profiles for tetrazomine and quinocarcin, illustrated in Figure 3, could therefore be explained by small differences in the pK_a's for the oxazolidine nitrogens. In other words, a slightly higher pH medium is necessary for deprotonation of the quinocarcin oxazolidine nitrogen, which subsequently turns on the redox cycle. Also supporting this notion is the fact that the NBT reductions for tetrazomine and quinocarcin exhibit the same overall trends.

Table 2: Cleavage of Supercoiled Plasmid DNA (pUC 19)

entry	conditions	[tetrazomine or quinocarcin]	S
1	20 mM phosphate, pH 5	1.0 mM tetrazomine	0.9
2	20 mM phosphate, pH 6	1.0 mM tetrazomine	4.4
3	20 mM phosphate pH 7	1.0 mM tetrazomine	9.0
4	20 mM phosphate, pH 8	1.0 mM tetrazomine	10.0
5	20 mM phosphate, pH 9	1.0 mM tetrazomine	10.0
6	20 mM phosphate, pH 8	1.0 mM quinocarcin	9.7
7	20 mM phosphate, pH 8	0.01 mM tetrazomine	0.0
8	20 mM phosphate, pH 8	0.1 mM tetrazomine	0.4

Table 3: Effects of Additives on Plasmid DNA Cleavage

entry	conditions[a]	[tetrazomine] (mM)	% inhibn	% enhancemt
1	0.1 mM $Fe^{II}SO_4$	1.0	0	0
2	0.1 mM $Fe^{III}NH_4SO_4$	1.0	5	
3	0.1 mM desferal	1.0	0	0
4	1.0 mM desferal	1.0	37	
5	10 mM desferal	1.0	94	
6	deoxygenated	1.0	80	
7	0.1 mM H_2O_2	0.1		68
8	0.1 mM H_2O_2	1.0		29
9	1.0 mM picolinic acid	1.0	28	
10	10 mM picolinic acid	1.0	71	
11	10 μg/mL catalase	1.0	55	
12	100 μg/mL catalase	1.0	54	
13	10 μg/mL SOD	1.0	94	

[a] All reactions were run in 20 mM phosphate buffer (pH 8.0).

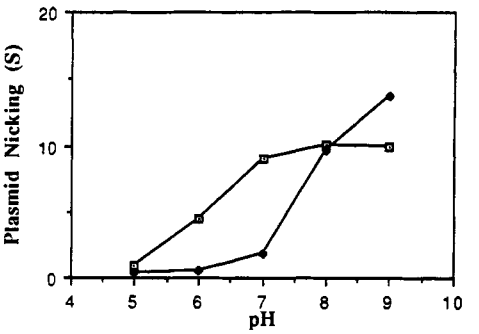

FIGURE 3: pH trends for plasmid nicking of tetrazomine (□) versus quinocarcin (◆). All reactions contained 0.15 μg of supercoiled plasmid DNA (pUC 19) and 1 mM drug and were run in 20 mM phosphate buffer at the indicated pH's (2 h, 37 °C). Values for S were calculated from the Poisson distribution, on the basis of the ratios of forms I (supercoiled), II (nicked open circular), and III (linear) of the DNA from scanning densitometry data of agarose electrophoresis gels following reactions.

Complete removal of oxygen from these experiments during the many manipulations is very difficult; however, partial exclusion of oxygen significantly inhibited this reaction, as expected (Table 3, entry 6). Superoxide dismutase also greatly inhibited DNA cleavage (Table 3, entry 13), which is consistent with both the capacity of tetrazomine to generate superoxide and the corresponding DNA cleavage event to be exclusively superoxide-dependent. Catalase inhibits the reaction, but not as potently as SOD (Table 3, entries 11 and 12). Addition of hydrogen peroxide to tetrazomine/DNA reactions had a potent stimulatory effect on DNA cleavage over control reactions containing hydrogen peroxide at the same concentrations (Table 3, entries 7 and 8). Taken together, the above

results point strongly to Fenton-type chemistry being responsible for the scission of DNA.

Tomita previously reported that the addition of metal ions such as Fe(III) had no stimulatory effect on the ability of quinocarcin to cleave DNA; we have corroborated this finding with tetrazomine, and the results appear in Table 3 (entries 1 and 2). The addition of the potent iron chelator desferal did, however, exhibit significant inhibition of DNA cleavage, particularly at high concentrations (Table III, entries 3–5). Desferal is known to have a high affinity for Fe(III) (log k_f = 30.7), forming a hexacoordinate complex that excludes iron-associated water and uncouples the oxidation of Fe(II) from the formation hydroxyl radical (Fenton reaction). The participation of higher oxidation states of iron and copper in Fenton reactions and related CH oxidation chemistry is now well-recognized (Saito et al., 1990; Zafirou & Bonneau, 1987). Picolinic acid is known (Sheu, 1990) to be a very potent scavenger of hydroxyl radical and inhibitor of the Fe(II)/Fe(III) redox couple. Addition of picolinic acid to reaction mixtures of DNA and tetrazomine at 1 and 10 mM showed 28% and 71% inhibition, respectively. The rate of formation of superoxide by tetrazomine (see Table 1) is extremely slow (10^4–10^5 times slower) relative to the rate-limiting step of the Haber-Weiss/Fenton reaction, which is 76 M^{-1} s^{-1} (Lesko, 1980) for the reduction of hydrogen peroxide by Fe(II). Thus, our data support a hypothesis wherein the limiting reagent in the DNA cleavage mediated by tetrazomine and quinocarcin is the slow production of superoxide. These results also support the notion that adventitious metal in these reaction mixtures in a number of possible oxidation states can be activated by the slow release of superoxide and cause Fenton-related damage to DNA. Thus, these results indicate that DNA cleavage is indeed metal-dependent and that the low concentration of adventitious Fe(III) present is already in excess of that required to effect Fenton-mediated cleavage of DNA; the addition of excess iron therefore would not be expected to have any additive effect. Thus, our data again support the hypothesis wherein the limiting reagent in the DNA cleavage mediated by quinocarcin or tetrazomine is the slow production of superoxide.

Reactions of Tetrazomine wtih 5′-^{32}P-Labeled Synthetic 45-mer for High-Resolution Polyacrylamide Gel Electrophoresis. Further evidence for a non-DNA-associated oxidant was obtained from analysis of the reaction of tetrazomine with a small synthetic oligonucleotide by high-resolution polyacrylamide gel electrophoresis. A synthetic 45-base-pair oligonucleotide, end-labeled with ^{32}P and annealed to its complement, was reacted with tetrazomine (0.1, 1.0, and 10 mM without any additional reducing agents) at 37 °C for 5 h (20 mM phosphate buffer, pH 8), resulting in non-sequence-specific cleavage at every nucleotide as evidenced by denaturing 20% polyacrylamide gel electrophoresis (Figure 4, lanes 3–5). Furthermore, every cleavage band appeared as a doublet, which is characteristic of the 3′-phosphate and 3′-phosphoglycolate ends resulting from nonselective Fenton-mediated cleavage (Tullius & Dombroski, 1985; Hertzberg & Dervan, 1984). We observed a similar cleavage pattern from incubating the duplex with 10 mM quinocarcin and with 1.0 mM $FeSO_4$/EDTA under aerobic conditions (Figure 4, lanes 6 and 7, respectively). Initially, we found that attenuated cleavage was observed at higher tetrazomine concentrations (10 mM) and was inconsistent with the results obtained by NBT reduction, which shows linear superoxide production over the same concentration range. One explanation for this observation was that in the presence of limited oxygen and high

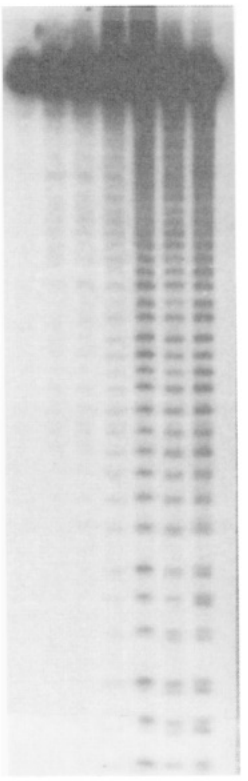

FIGURE 4: Lane 1: 5′-^{32}P-labeled duplex 45-mer. Lane 2: DNA (control). Lane 3: DNA + 0.1 mM tetrazomine. Lane 4: DNA + 1.0 mM tetrazomine. Lane 5: DNA + 10 mM tetrazomine. Lane 6: DNA + 10 mM quinocarcin. Lane 7: DNA + 1.0 mM Fe^{II}/EDTA. There was no significant evidence for any sequence specificity to the cleavage of this substrate (sequence along the gel is therefore superfluous and has been deleted).

tetrazomine concentrations a large portion of the hydroxyl radical produced was not in close proximity to the DNA, therefore exhausting the oxygen source before significant damage to DNA could occur. When reaction mixtures are saturated with oxygen, however, a more linear rate of DNA damage with respect to tetrazomine concentration is observed, as illustrated in Figure 4. An additional factor for this observation could be that tetrazomine, noncovalently or covalently associated within the minor groove of DNA, may protect the DNA from its own oxidative damaging potential. It has been proposed through molecular modeling (Hill et al., 1988) that quinocarcin docks in the minor groove and may alkylate DNA through the ring-opened iminium form (which would be analogous to **5**). The effect of tetrazomine associated to DNA in a similar manner might, therefore, be to protect the ribose phosphate backbone from oxidative damage by blocking sites in the minor groove. Evidence for the latter comes from experiments that show an increased resistance to Fe/EDTA cleavage for DNA that has first been incubated with tetrazomine (10 mM) for 5 h followed by ethanol precipitation (Figure 5, lane 3). However, when the order of reagents is reversed (i.e., treated with Fe/EDTA followed by 10 mM tetrazomine), significantly more cleavage is observed consistently (Figure 5, lane 4).

Another noteworthy observation is the change in relative ratios of the two 3′-base-pair products (phosphate versus phosphoglycolate) observed when comparing tetrazomine to quinocarcin and Fe/EDTA (Figures 4 and 6). By scanning densitometry, the relative ratios for phosphate to phosphoglycolate are 8:2 for tetrazomine, 6:4 for quinocarcin, and 4:6 for Fe/EDTA (lanes 5–7, respectively). Although speculative,

FIGURE 5: Lane 1: 5′-^{32}P-labeled duplex 45-mer. Lane 2: DNA (control). Lane 3: DNA + 10 mM tetrazomine followed by 1.0 mM Fe^{II}/EDTA. Lane 4: DNA + 1.0 mM Fe^{II}/EDTA followed by 10 mM tetrazomine. All reactions were carried out in phosphate buffer (pH 8) for 5 h with ethanol precipitations carried out between subsequent reactions.

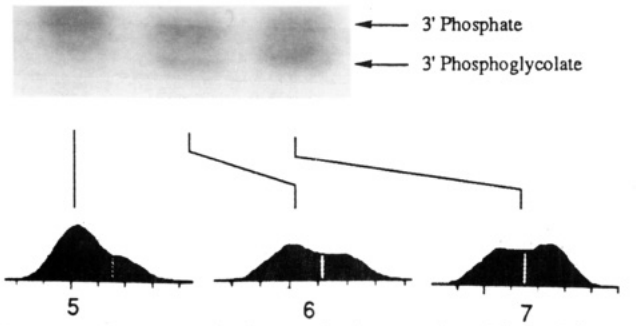

FIGURE 6: Representative base-pair cleavages from Figure 4 (lanes 5–7). Ratios of 3′-phosphate to 3′-phosphoglycolate by scanning densitometry are 8:2, 6:4, and 4:6 for tetrazomine (5), quinocarcin (6), and Fe/EDTA (7), respectively.

these results point to this protective behavior that these compounds exhibit, at least as far as abstraction of the 4′-hydrogen, which ultimately leads to the 3′-phosphoglycolate product, is concerned. This is again supported by the results illustrated in Figure 5, which show virtually no 3′-phosphoglycolate formation upon the treatment of DNA with Fe/EDTA that has first been incubated with tetrazomine.

DISCUSSION

Superoxide production by quinocarcin was previously reported (Tomita et al., 1984), and a mechanistic explanation has now been offered (Williams et al., 1992). Structural determination for tetrazomine (Sato et al., 1991) has revealed a ring system similar to that of quinocarcin, although little is currently known about the stereochemistry of this compound. On the basis of the analogous aerobic behavior that tetrazomine shares with quinocarcin, we now propose the same relative stereochemical configuration of quinocarcin for tetrazomine (with respect to the oxazolidine moiety). This assertion is based on studies (Williams et al., 1991) involving synthetic analogs **11** and **12** in Figure 1 (three-dimensional structures based on X-ray analysis), which suggest the quinocarcin oxazolidine configuration to be necessary for this redox mechanism to operate. When **11**, which has the same configuration as quinocarcin (*syn*), is allowed to stand in phosphate buffer (pH 8) under aerobic conditions at room temperature, it spontaneously produces superoxide (Table 1, entry 7). Under the same conditions, **12**, which is in the *anti* (non-quinocarcin) configuration, does not generate superoxide (Table 1, entry 8). Therefore, since tetrazomine readily produces superoxide under these conditions, this suggests that it possesses the same relative stereochemical configuration illustrated by **11** and quinocarcin, where the nitrogen lone pair is *trans*-antiperiplanar to the adjacent oxazolidinyl methine. This appears to be a requirement for redox chemistry to proceed on the basis of our observations.

The capacity of many antitumor antibiotics to cause oxidative damage to DNA in cancerous tissues is typically inseparable from the nonspecific damage inflicted on healthy cells by these reduced oxygen species and is widely recognized to be associated with the undesirable host toxicity of most antitumor drugs. The recognition of new mechanisms for the production of such reactive oxygen species and the chemical means to attenuate this reactivity without compromising other modes of action often displayed by such substances, such as nucleic acid alkylation, intercalation, and DNA polymerase inhibition, will be essential to designing more specific and efficacious cancer chemotherapeutic agents.

In summary, we have presented another example of a previously unrecognized reaction for the reduction of molecular oxygen by a simple heterocyclic ring system. This reduction is driven by the inherent intermolecular redox chemistry of the drug itself, requiring no exogenous reductants. Chemical means to attenuate the ability of this class of antitumor drugs to produce reactive oxidants (**11** versus **12**, **10**, stabilizers such as citric acid, stimulators such as DTT and hydrogen peroxide) may contribute to possible approaches to designing more selective and less toxic cancer chemotherapeutic agents. Studies aimed at elucidating the details of the expected covalent interactions of this class of compounds with nucleic acid targets are underway. In addition, further mechanistic studies employing synthetic analogs of these compounds are underway in these laboratories.

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