

# DNA Cleavage by the Antitumor Agent 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (SR4233): Evidence for Involvement of Hydroxyl Radical

J. Scott Daniels<sup>†</sup> and Kent S. Gates<sup>\*,†,‡</sup>

Contribution from the Departments of Chemistry and Biochemistry, University of Missouri–Columbia, Columbia, Missouri 65211

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**Abstract:** 3-Amino-1,2,4-benzotriazine 1,4-dioxide (SR4233, WIN59075, tirapazamine, **1**) is a clinically promising antitumor agent that requires bioreductive activation, selectively kills oxygen-deficient cells, and is believed to derive its biological activity from DNA cleavage. Using a xanthine–xanthine oxidase enzyme system as a one-electron reductant to activate **1** for DNA cleavage, it has been found that radical scavengers such as mannitol, dimethyl sulfoxide, ethanol, methanol, and *tert*-butyl alcohol significantly inhibit drug-dependent DNA cleavage. Compound **1**, in concert with the xanthine–xanthine oxidase system, converts DMSO to methanesulfinic acid, a reaction characteristic of hydroxyl radical. In addition, treatment of a <sup>32</sup>P-labeled restriction fragment with reductively-activated **1** results in cleavage at every base pair, with little sequence specificity, consistent with involvement of a highly reactive, nonselective agent such as hydroxyl radical. These results strongly support the involvement of radicals in the cleavage of DNA by **1** and are consistent with hydroxyl radical as the major DNA-cleaving species generated by reduction of **1**.

Compounds that damage DNA play an important role in cancer chemotherapy.<sup>1</sup> In the pursuit of improved cancer chemotherapeutic agents, one approach involves identification of features unique to cancer cells that can be used to direct the cytotoxic action of DNA-damaging agents specifically toward these cells. One such feature that may be exploited in the treatment of certain cancers is the oxygen-deficient (hypoxic) nature of solid tumor cells relative to normal cells.<sup>2</sup> Due to the fact that hypoxic cells are resistant to radiation therapy<sup>3</sup> and a number of common chemotherapeutic agents,<sup>4</sup> tumor cell hypoxia is often a problem rather than an advantage in cancer treatment; however, several promising or clinically useful antitumor agents are thought to obtain some therapeutic advantage by causing DNA damage more efficiently in hypoxic tumor cells as compared to normally oxygenated cells.<sup>5</sup>

1,2,4-Benzotriazine 1,4-dioxides are a novel class of anti-cancer agents whose remarkable antitumor properties are thought to stem from their selective toxicity toward the hypoxic cells found in solid tumors.<sup>6–8</sup> One member of this class of molecules, 3-amino-1,2,4-benzotriazine 1,4-dioxide (SR4233, WIN 59075, tirapazamine, **1**), is currently in phase II and III clinical trials for the treatment of certain cancers.<sup>8</sup> Because it is thought that **1** derives its biological activity from the cleavage of cellular DNA,<sup>6–8</sup> we undertook an investigation of the mechanism of DNA cleavage by **1** with the expectation that a detailed understanding of this chemistry might ultimately facilitate the design of new therapeutic agents with improved antitumor properties.

It is believed that *in vivo* DNA cleavage by **1** is due to a radical species generated by enzymatic one-electron reduction of the heterocycle (Scheme 1).<sup>8</sup> This theory is supported by several observations. In the absence of reducing systems, **1** alone does not damage DNA.<sup>9,10</sup> In mammalian cells under anaerobic conditions, **1** is ultimately reduced to **3**, which is not highly cytotoxic,<sup>6,11</sup> and the rates of reduction parallel cytotoxicity in several different cell lines.<sup>12,13</sup> Furthermore, Brown and co-workers have shown that addition of the radical scavenger dimethyl sulfoxide (DMSO) to hypoxic cell cultures significantly

\* Author to whom correspondence should be addressed.

<sup>†</sup> Department of Chemistry.

<sup>‡</sup> Department of Biochemistry.

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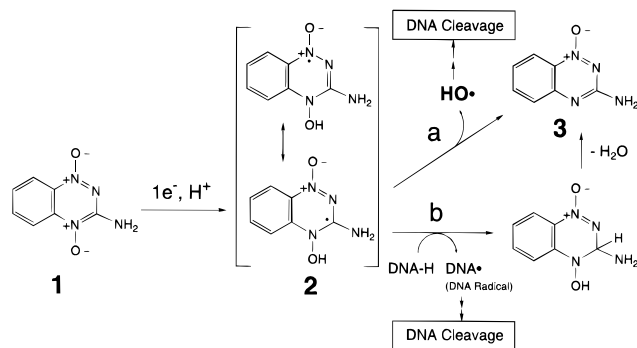
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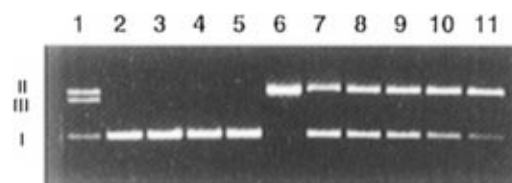
**Scheme 1.** Two Possible Mechanisms for DNA Cleavage by **1**

reduces the cytotoxicity of **1**.<sup>8,14</sup> A radical species resulting from the incubation of **1** with rat liver microsomes has been observed by ESR;<sup>15</sup> however, no relation between this radical and DNA cleavage has been established. The identity of the enzyme(s) responsible for *in vivo* reductive activation of **1** remains a subject of investigation.<sup>9,16</sup> The specific toxicity of **1** toward hypoxic cells may result from the fact that the “activated” radical form of the drug (**2**) is destroyed by reaction with molecular oxygen.<sup>17</sup> Such a back-oxidation would regenerate the drug (**1**) and produce superoxide radical, a species whose *in vivo* toxicity is mitigated by the cellular enzymes superoxide dismutase and catalase.<sup>18</sup> Here we report evidence strongly supporting the notion that radical species are involved in the cleavage of DNA by reductively-activated **1**. In addition, we provide evidence that hydroxyl radical, rather than a radical form of **1**, may be the major DNA-cleaving species in these reactions.

**Results**

Two possible pathways for DNA cleavage by one-electron reduced **1** under anaerobic conditions are shown in Scheme 1.<sup>11,17</sup> Pathway **a** involves decomposition of the protonated radical **2** to produce the observed metabolite **3** and hydroxyl radical, a known DNA-cleaving agent.<sup>19</sup> A second possibility (pathway **b**, Scheme 1) involves direct abstraction of hydrogen atoms from the sugar–phosphate backbone of DNA by drug radical **2**, followed by dehydration to yield **3**. Abstraction of hydrogen atoms from the deoxyribose backbone of DNA is known to result in strand scission.<sup>19,20</sup>

**DNA Cleavage by 1.** Using a xanthine–xanthine oxidase enzyme system<sup>21,22</sup> for the reduction of **1**<sup>24</sup> under anaerobic conditions (Scheme 2), we have confirmed that, upon reduction, this di-*N*-oxide (250  $\mu$ M) efficiently cleaves DNA (lane 6,



**Figure 1.** Cleavage of supercoiled plasmid DNA by 3-amino-1,2,4-benzotriazine 1,4-dioxide (**1**). All reactions contain DNA, buffer, xanthine oxidase, desferal, superoxide dismutase, and catalase and were performed under anaerobic conditions. Lane 1: marker lane containing a mixture of supercoiled (form I), nicked (form II), and linear (form III) pBR322. Lane 2: xanthine oxidase–xanthine alone (no **1** or **3**). Lane 3: **3** (250  $\mu$ M, 10% acetonitrile) + xanthine oxidase (no xanthine). Lane 4: **3** (250  $\mu$ M, 10% acetonitrile) + xanthine oxidase–xanthine. Lane 5: **1** (250  $\mu$ M) + xanthine oxidase (no xanthine). Lane 6: **1** (250  $\mu$ M) + xanthine oxidase–xanthine. Lanes 7–11: **1** (250  $\mu$ M) + xanthine oxidase–xanthine and mannitol (100 mM, lane 7), dimethyl sulfoxide (100 mM, lane 8), ethanol (100 mM, lane 9), methanol (100 mM, lane 10), and *tert*-butyl alcohol (100 mM, lane 11).

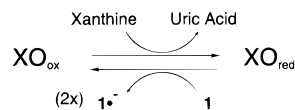
**Scheme 2.** Reduction of **1** by Xanthine Oxidase (XO) Under Anaerobic Conditions

Figure 1).<sup>9,21</sup> DNA cleavage absolutely depends on enzymatic reduction of **1**; no significant DNA cleavage is observed if **1** or xanthine is omitted from the reaction mixture (lanes 2 and 5).<sup>25</sup> As expected, the two-electron reduced form of **1** (mono-*N*-oxide **3**,<sup>24</sup> 250  $\mu$ M) alone is not a DNA-cleaving agent (lane 3). The mono-*N*-oxide **3** (250  $\mu$ M) does, however, produce very weak reduction-dependent DNA cleavage when incubated with the xanthine–xanthine oxidase enzyme system (Table 1).<sup>26</sup> Under conditions of ambient oxygenation, little or no enzyme-activated DNA cleavage by **1** is observed (Table 1). The observed inhibition of DNA cleavage by dissolved oxygen is consistent with a molecular oxygen-dependent back-oxidation of the radical **2**.<sup>17</sup> We find that reducing agents such as sodium dithionite, sodium ascorbate, and thiols are incapable of effecting reductive activation of **1** (data not shown).

Importantly, we find that commonly used radical scavengers such as mannitol, DMSO, ethanol, methanol and *tert*-butyl

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(22) The xanthine–xanthine oxidase enzyme system is known to produce superoxide radical under aerobic conditions.<sup>18</sup> Unless otherwise noted, all assays described herein were performed under “anaerobic” conditions. Although significant efforts were taken to remove and exclude molecular oxygen (see Experimental Section) the reaction mixtures undoubtedly contain significant amounts of dissolved oxygen and, thus, might best be described as “low oxygen” rather than anaerobic. To suppress background DNA-cleavage resulting from xanthine oxidase-mediated conversion of trace molecular oxygen to superoxide, we employ desferal (desferrioxamine mesylate) or diethylenetriaminepentaacetic acid to sequester traces of adventitious iron in a non-redox-active form, thus inhibiting the conversion of superoxide radical to DNA-cleaving agent hydroxyl radical.<sup>23</sup> These agents were used at moderate concentrations (1–10 mM), below the level where they would be expected to efficiently scavenge radical species. In addition, the reactions contain superoxide dismutase and catalase to decompose any traces of superoxide radical or hydrogen peroxide.<sup>18</sup>

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(26) The weak cleavage of DNA by **3** in our *in vitro* assays is consistent with its weak biological activity.<sup>11</sup> Poor cleavage by **3** may be due primarily to the fact that the compound is not efficiently reduced by the xanthine–xanthine oxidase enzyme system used in these studies (J. S. Daniels, K. S. Gates, unpublished data); however, we suggest that the small amount of observed DNA cleavage by the mono-*N*-oxide **3** may proceed by a hydroxyl radical-liberating mechanism analogous to pathway **a** in Scheme 1.

**Table 1.** Effect of Various Conditions and Additives on the Cleavage of Plasmid DNA by **1**<sup>a</sup>

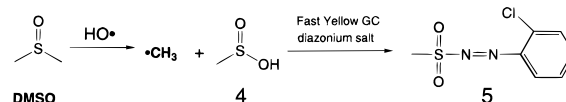
reaction	% form I	% form II	% form III
250 $\mu$ M <b>1</b> + XO <sup>b</sup>	97	3	
X/XO alone, anaerobic	94	6	
standard reaction: <sup>a</sup> 250 $\mu$ M <b>1</b> + X/XO, anaerobic	12	83	5
standard reaction except: MES buffer (pH 6.5, 50 mM)	61	40	
standard reaction except: HEPES buffer (pH 7.2, 50 mM)	65	35	
standard reaction except aerobic	90	10	
X/XO alone, aerobic	90	10	
standard reaction except: 250 $\mu$ M <b>3</b> (instead of <b>1</b> )	84	16	
standard reaction—plus scavengers			
mannitol (100 mM)	57	43	
DMSO (100 mM)	50	50	
ethanol (100 mM)	44	56	
methanol (100 mM)	46	54	
<i>tert</i> -butyl alcohol (100 mM)	34	66	
glutathione (100 mM)	22	78	

<sup>a</sup> The standard cleavage reaction contains supercoiled pBR322 DNA (12  $\mu$ g/mL), **1** (250  $\mu$ M), xanthine (250  $\mu$ M), desferal (1 mM), superoxide dismutase (10  $\mu$ g/mL), catalase (100  $\mu$ g/mL), and xanthine oxidase (0.4 units/mL) in sodium phosphate buffer (50 mM, pH 7.0). Reactions and densitometry performed as described in the Experimental Section. Values reflect the average of multiple experiments. The standard error of the reported values is approximately 5%. <sup>b</sup> X/XO = xanthine/xanthine oxidase.

alcohol significantly inhibit DNA cleavage by xanthine oxidase-reduced **1** (lanes 7–11, Figure 1, Table 1). Control experiments show that the radical scavengers used in our studies do not inhibit reduction of **1** by the xanthine–xanthine oxidase system. These results clearly implicate radical intermediates in the xanthine oxidase-mediated cleavage of DNA by **1**. Furthermore, the radical scavengers used in our experiments are thought to react *specifically* with oxygen radicals versus other radical species,<sup>27,28</sup> thereby suggesting that hydroxyl radical (pathway **a**, Scheme 1) may be involved in the cleavage of DNA by **1**.

While pH has little effect on this DNA-cleavage system (data not shown), the identity of the buffer salt used has a marked effect. Relative to sodium phosphate buffer, we find diminished DNA cleavage in buffers such as 4-morpholineethanesulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), consistent with the known ability of these organic buffers to react with hydroxyl radical (Table 1).<sup>28,29</sup>

**Conversion of DMSO to Methanesulfinic Acid by Reductively-Activated **1**.** Additional evidence implicating hydroxyl radical in DNA cleavage by **1** is provided by our finding that **1**, in combination with the xanthine–xanthine oxidase system under anaerobic conditions, converts DMSO to methanesulfinic acid (**4**). This is a reaction characteristic of hydroxyl radical (Scheme 3).<sup>30</sup> The methanesulfinic acid produced in these reactions was detected as the diazosulfone (**5**) that results from

**Scheme 3.** Reaction of Hydroxyl Radical with DMSO and Subsequent Formation of the Diazosulfone Derivative of Methanesulfinic Acid**Table 2.** Detection of Methanesulfinic Acid (MSA) Produced in the Reaction of Reductively Activated **1** with DMSO

assay <sup>a</sup>	HPLC peak height <sup>b</sup> for MSA-diazosulfone ( <b>5</b> )
X/XO <sup>c</sup> alone, no <b>1</b>	6851
<b>1</b> + XO, no X	2153
<b>1</b> + X/XO	43022
<b>calibration assays<sup>a</sup></b>	
0.02 $\mu$ mol MSA (2 mL of 10 $\mu$ M soln)	2272
0.1 $\mu$ mol MSA (2 mL of 50 $\mu$ M soln)	18957
0.6 $\mu$ mol MSA (2 mL of 300 $\mu$ M soln)	41192
0.8 $\mu$ mol MSA (2 mL of 400 $\mu$ M soln)	37966
1 $\mu$ mol MSA (2 mL of 500 $\mu$ M soln)	64123

<sup>a</sup> MSA detection assays performed as described in the Experimental Section. Standard error in the measurement of MSA is approximately 15%. <sup>b</sup> Relative peak heights reported for HPLC detection of the MSA-diazosulfone (**5**) are in arbitrary units. <sup>c</sup> X/XO = xanthine/xanthine oxidase.

derivatization with the aromatic diazonium salt, Fast Yellow GC (Scheme 3).<sup>31,32</sup> Incubation of DMSO with the xanthine–xanthine oxidase enzyme system, under identical conditions in the absence of **1**, produces only small amounts of methanesulfinic acid. If DMSO is omitted from the reaction mixtures, little methanesulfinic acid signal is observed (Table 2).

Conversion of DMSO to methanesulfinic acid by the **1**/xanthine–xanthine oxidase system is a reasonably efficient process. Calibration curves obtained by quantitative detection of known amounts of methanesulfinic acid allow estimation of the amount of drug-dependent oxidation of DMSO to methanesulfinic acid in this system. Enzymatic reduction of 1  $\mu$ mol of **1** (2 mL of a 500  $\mu$ M solution) by the xanthine–xanthine oxidase system results in the production of more than 0.5  $\mu$ mol (2 mL of a >250  $\mu$ M solution) of methanesulfinic acid, with background methanesulfinic acid production subtracted out (Table 2).

**Sequence Specificity of DNA Cleavage by **1**.** Our hypothesis that hydroxyl radical is responsible for the observed DNA cleavage by **1** predicts that this agent should display little sequence specificity other than that which results from sequence-dependent changes in the shape of double-helical DNA.<sup>33</sup> In order to investigate this question, we compared the cleavage of a 377 base pair DNA fragment by **1** to that by an iron–EDTA system. The iron–EDTA system used for comparison has recently been shown to produce a DNA-cleavage pattern identical to that of hydroxyl radical which was generated by  $\gamma$ -radiolysis.<sup>29,34</sup> We find that reductively-activated **1** cleaves DNA at every base pair, with little sequence dependence or base

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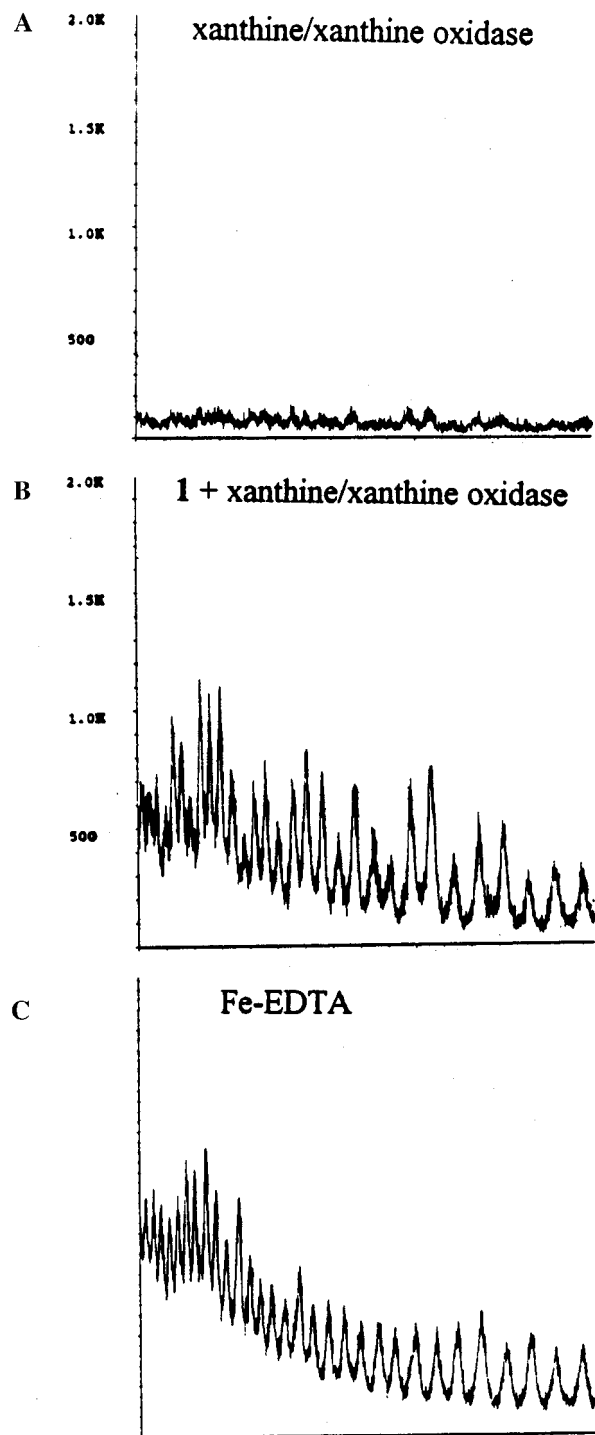
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**Figure 2.** Comparison of DNA-cleavage patterns generated by: (A) control; xanthine–xanthine oxidase enzyme system; (B) compound **1** activated by xanthine–xanthine oxidase; and (C) a hydroxyl radical-generating Fe–EDTA system.<sup>29</sup> DNA cleavage reactions were performed on a 377 base pair <sup>32</sup>P-labeled restriction fragment as described in the Experimental Section. Densitometer scans are from a portion of a 20% denaturing polyacrylamide gel and show the relative intensity of DNA cleavage at each base position. Lanes A and B were loaded with equal amounts (cpm) of labeled DNA; lane C, provided for comparison, is not plotted on the same y-axis scale as A and B. The densitometer scans are aligned horizontally.

specificity (Figure 2). It is not yet clear whether the moderate sequence preferences observed in this cleavage reaction derive from weak association of the activating enzyme xanthine oxidase

with double-helical DNA or from association of the drug with DNA prior to or following enzymatic reduction. Regardless, the low sequence selectivity observed in the DNA-cleavage reaction of **1** is consistent with involvement of a highly reactive, nonselective cleaving agent such as hydroxyl radical.

## Discussion

Our results suggest that hydroxyl radical is formed in a reaction that is triggered by one-electron enzymatic reduction of the promising antitumor agent **1**. Efficient inhibition of DNA cleavage by DMSO and alcohols is consistent with the intermediacy of oxygen-centered radicals.<sup>37</sup> Ethanol, for example, is known to rapidly quench hydroxyl radical,<sup>28</sup> but is a relatively inefficient scavenger of some carbon-centered radicals.<sup>27</sup> In addition, we have shown that the reactive intermediate generated by the action of xanthine–xanthine oxidase on **1** converts DMSO to methanesulfinic acid, a reaction considered characteristic and diagnostic of hydroxyl radical.<sup>30–32</sup> Finally, we have demonstrated that the DNA-cleaving species generated by reductive activation of **1** cleaves DNA with nearly neutral sequence specificity, similar to that observed for hydroxyl radical.<sup>29</sup>

When considered together, our results suggest that enzymatic one-electron reduction of the antitumor agent **1** leads to efficient production of hydroxyl radical (pathway **a**, Scheme 1). The redox-activated DNA cleavage by **1** is markedly inhibited by molecular oxygen. Thus, it appears that **1**, in concert with reductive cellular enzymes, may serve as a vehicle to deliver the known DNA-cleaving agent of radiotherapy, hydroxyl radical,<sup>38</sup> specifically to hypoxic tumor cells *in vivo*.<sup>39</sup>

The mechanism supported by our data (pathway **a**, Scheme 1) differs from that which is commonly put forward to explain reductively-activated DNA cleavage by **1**—that is, direct hydrogen atom abstraction from DNA by the carbon-centered radical **2** (pathway **b**, Scheme 1).<sup>8,9,40</sup> The mechanism we favor is analogous to that suggested by Hecht and co-workers to explain redox-activated DNA cleavage by phenazine *N*-oxides such as **6**.<sup>41</sup> The superiority of pathway **a** over pathway **b** can be rationalized by noting that formation of the high-energy hydroxyl radical from **2** may be thermodynamically driven by rearomatization of the triazine ring system and by the entropically favorable fragmentation. A similar driving force is not evident for pathway **b**, where a high-energy radical would be formed in the direct reaction of **2** with a hydrogen on the DNA backbone. Radical fragmentation reactions thought to be thermodynamically driven by the formation of aromatic or conjugated molecules are common in organic chemistry.<sup>42</sup> For example, it is well-known that *N*-substituted pyridine-2(1*H*)-thiones undergo radical or photoinduced fragmentation reactions in which the pyridine-2(1*H*)-thione heterocycle is aromatized while a reactive radical is released. A variety of radicals,

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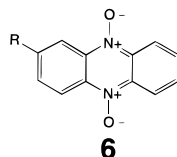
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including hydroxyl radical, have been generated using the pyridine-2(1*H*)-thione methodology.<sup>43</sup>



Our results, coupled with previous work by Hecht and co-workers describing DNA cleavage by phenazine *N*-oxides,<sup>41</sup> suggest the possibility that reduction-dependent fragmentation reactions resulting in the production of hydroxyl radical may obtain for a number of structurally diverse *N*-oxide antitumor, antibacterial, and antifungal agents<sup>44–47</sup> whose mode of action has been suggested to involve DNA damage. We are currently investigating this and other aspects of DNA cleavage by *N*-oxides.

## Experimental Section

**Reagents.** Materials were purchased from the following suppliers and were of the highest purity available: xanthine, D-mannitol, DMSO, L-ascorbic acid, ethylenediaminetetraacetic acid (EDTA), glutathione, MES, sodium dithionite, thiourea, sodium phosphate, diethylenetriaminepentaacetic acid (DETAPAC), Aldrich Chemical Co.; sodium acetate, HEPES, Fast Yellow GC salt, glycerol, tris(hydroxymethyl)aminomethane (Tris), G-50 Sephadex, *N,N'*-methylenebisacrylamide, and boric acid, Sigma Chemical Co.; methanesulfinic acid, Lancaster; hydrogen peroxide, iron chloride, HPLC grade solvents (ethyl acetate, methanol, acetonitrile, hexane, 2-propanol), and *tert*-butyl alcohol, Fisher; Klenow fragment of DNA polymerase I, bovine serum albumin, EcoRI, and BamHI, New England Biolabs; 2'-deoxynucleoside-5'-triphosphates, Pharmacia; acrylamide, ethidium bromide, xanthine oxidase, catalase, and superoxide dismutase, Boehringer Mannheim; Seakem ME agarose, FMC; ethanol, McCormick Distilling Co., Inc.; urea, xylene cyanol, bromophenol blue, and sodium dodecyl sulfate (SDS), United States Biochemical; desferal was a generous gift from Ciba-Geigy Co.; 5'-[ $\alpha$ -<sup>32</sup>P]dATP, New England Nuclear-DuPont; pBR322 supercoiled plasmid DNA was isolated from *E. coli* and purified by cesium chloride gradient centrifugation<sup>48</sup> or purchased from Boehringer Mannheim. 3-Amino-1,2,4-benzotriazine 1,4-dioxide (**1**) and 3-amino-1,2,4-benzotriazine 1-oxide (**3**) were prepared according to the methods of Mason and Tennant<sup>24</sup> and all spectral data and melting points agree with those reported in the literature. UV-vis spectra were recorded using a Hewlett-Packard 8452A instrument; densitometry was performed using a Zeineh SL-TRFF scanning laser densitometer or an Alpha Innotech IS-1000 digital imaging system.

**Cleavage of Supercoiled Plasmid DNA.** Unless noted otherwise, all assays were performed under anaerobic conditions and were carried out in a glovebox or glovebag purged with pre-purified nitrogen. Individual components of the assays were degassed by bubbling nitrogen through each solution for 1 minute. In a typical DNA cleavage

assay (final volume 50  $\mu$ L), to a degassed solution containing supercoiled pBR322 (600 ng), xanthine (250  $\mu$ M, added as a solution in 40% aqueous NaOH), desferal (1 mM), superoxide dismutase (10  $\mu$ g/mL), catalase (100  $\mu$ g/mL), and **1** (250  $\mu$ M) in sodium phosphate (50 mM, pH 7.0) was added 0.02 unit of xanthine oxidase. The reactions were capped, vortex mixed, removed from the glovebag, and incubated at 24 °C for 1 h. Reactions involving **3** contained 10% acetonitrile as a cosolvent.

Following incubation, 5  $\mu$ L of 50% glycerol loading buffer<sup>48</sup> containing 0.1% bromophenol blue, 150 mM EDTA, 1% SDS in 2 M Tris, 1 M acetate, pH 8 was added to the reactions and the resulting mixture loaded immediately onto a 0.9% agarose gel. The gel was electrophoresed for approximately 4 h at 80 V in 1  $\times$  TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8) buffer and then stained in an aqueous ethidium bromide solution (0.3  $\mu$ g/mL) for 1–2 h. DNA in the gel was visualized by UV-transillumination and the gel photographed using either Polaroid 665 or 55 film. The amount of DNA in each band of the ethidium-stained gels was quantitated from Polaroid 55 negatives by scanning laser densitometry or by digital imaging using an Alpha Innotech IS-1000 system. The values reported are uncorrected for differential ethidium staining of form I and II DNA.<sup>49</sup>

**Inhibition of DNA Cleavage by Radical Scavengers.** DNA cleavage reactions containing radical scavengers were performed as described above except the scavenging agent was added to the reaction mixture prior to addition of xanthine oxidase. In order to demonstrate that diminished DNA cleavage resulting from the addition of radical scavenging agents such as DMSO was not due to inhibition of xanthine oxidase-catalyzed reduction of **1**, the disappearance of **1** under conditions analogous to the DNA-cleavage reactions was directly monitored in the presence and absence of scavenging agents using HPLC. Compound **1** (monitored at 266 nm) has a retention time of approximately 6 min using a Rainin Dynamax phenyl column eluted with methanol/acetonitrile/water (1:2:7). By this method it was found that radical scavengers have a negligible effect on the reduction of **1** by xanthine oxidase. In addition, we find that radical scavengers have no effect on the rate of xanthine oxidation by xanthine oxidase, as determined by monitoring the production of uric acid at 294 nm.<sup>50</sup>

**Methanesulfinic Acid Detection.** Methanesulfinic acid produced by the oxidation of DMSO was detected and quantitated using a modified version of the protocol reported by Fukui et al.<sup>31</sup> In a typical assay, under anaerobic conditions as described above, to a degassed solution containing **1** (500  $\mu$ M), xanthine (1 mM), DETAPAC or desferal (1 mM), and DMSO (500 mM), in sodium phosphate (50 mM, pH 7.0), was added 0.3 unit of xanthine oxidase. The reaction (2 mL final volume) was capped, vortex mixed, and allowed to incubate at 24 °C for 1 h. Sodium phosphate (1 mL, 500 mM, pH 4.0) was added to the reaction, followed by Fast Yellow GC diazonium salt (1 mL of an approximately 10 mg/mL, 0.45  $\mu$ m-filtered solution) and the mixture allowed to stand at room temperature for 10 min. The resulting yellow solution was extracted with ethyl acetate (2 mL) and exactly 1.2 mL of the upper ethyl acetate layer removed by pipet. A portion of the extract (0.6 mL) was evaporated under reduced pressure at 30 °C and redissolved in 100  $\mu$ L of ethyl acetate. A portion of this ethyl acetate solution (20  $\mu$ L) containing the methanesulfinic acid diazosulfone (**5**) was then analyzed by HPLC. The diazosulfone conjugate (**5**, monitored at 285 nm) has a retention time of approximately 7 min on a Rainin Microsorb-MV propylamine column eluted with hexane–2-propanol (10:3).

Calibration curves for the detection of methanesulfinic acid by this method were constructed by dissolving known amounts of methanesulfinic acid in sodium phosphate (50 mM, pH 7.0, 2 mL final volume, containing no xanthine, xanthine oxidase, or **1**) and subjecting the solution to treatment as described above. The amount of **5** resulting from each assay was quantitated by measuring HPLC peak height or area.

**Preparation of a 3'-<sup>32</sup>P-End-Labeled 377 Base Pair DNA Restriction Fragment.** Plasmid pBR322 DNA was digested with EcoRI and 3'-end labeled using the Klenow fragment of DNA polymerase I.<sup>48</sup> A

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second restriction enzyme digest was performed with BamHI and the desired 3'-labeled 377 base pair fragment isolated using gel electrophoresis on a 5% polyacrylamide gel.

**Cleavage of the 3'-Labeled DNA Fragment by Fe-EDTA.**

Cleavage of DNA by the iron-EDTA system was performed as described by Pogozelski et al.<sup>28</sup> Following incubation, the reactions were ethanol precipitated and the precipitate was briefly dried under vacuum, redissolved in formamide loading buffer (10  $\mu$ L),<sup>48</sup> heated for 5 min at 90 °C, and then loaded onto a 20% denaturing polyacrylamide sequencing gel (1:19 cross-linked, 0.4 mm thick, containing 7.5 M urea) and electrophoresed for 14 h at 1600 V in 1  $\times$  TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8).

**Cleavage of the 3'-Labeled DNA Fragment by 1.** DNA-cleavage reactions involving **1** were degassed and performed in a glovebag as described above. To a degassed solution (50  $\mu$ L final volume) containing the 3'-labeled 377 base pair restriction fragment (100 000 cpm), **1** (5 mM), xanthine (5 mM), desferal (1 mM), superoxide dismutase (10  $\mu$ g/mL), and catalase (100  $\mu$ g/mL) in sodium phosphate (50 mM, pH 7.0) was added 0.2 unit of xanthine oxidase. The reactions were capped, vortexed, and incubated for 1 h at 24 °C. The reactions were then phenol extracted, desalted through a Sephadex G-50 spin column,<sup>48</sup> ethanol precipitated, briefly dried under vacuum, and redissolved in formamide loading buffer (10  $\mu$ L). The samples were

heated for 5 min at 90 °C and loaded onto a 20% denaturing polyacrylamide gel (1:19 cross-linked, 0.4 mm thick, containing 7.5 M urea) and the gel was electrophoresed at 1600 V for 14 h in 1  $\times$  TBE buffer. Following electrophoresis, radioactivity on the gels was imaged using Fuji RX X-ray film. Alternatively, radioactivity was visualized by exposing a PhosphorImager plate to the gel, followed by scanning of the plate using a Molecular Dynamics Model 400E PhosphorImager (Sunnyvale, CA).

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