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Enzyme-Activated, Hypoxia-Selective DNA Damage by 3-Amino-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxide

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The compound 3-amino-2-quinoxalinecarbonitrile 1,4-dioxide (**4**) displays potent hypoxia-selective cytotoxicity in cell culture. This compound is structurally similar to the known hypoxia-selective DNA-damaging agent tirapazamine (**1**, TPZ), but the ability of **4** to cause DNA damage under low-oxygen conditions has not previously been characterized. The results presented here provide the first evidence that **4** causes reductively activated DNA damage under hypoxic conditions. The findings indicate that one-electron reduction of **4** by NADPH:cytochrome P450 reductase yields an oxygen-sensitive intermediate (**5**). This activated intermediate is rapidly destroyed by reaction with O_2 under aerobic conditions, but goes forward to cause DNA damage under low-oxygen conditions. Analysis of the DNA damage indicates that reductive activation of **4** leads to production of a highly reactive, freely diffusible oxidizing radical that causes sequence-independent cleavage of the deoxyribose backbone and oxidative damage to the heterocyclic bases in duplex DNA. On the basis of the experiments reported here, the chemical nature of the DNA damage caused by redox-activated **4** is analogous to that reported previously for TPZ.

Introduction

The compound 3-amino-1,2,4-benzotriazine 1,4-*N*-oxide (**1**, tirapazamine, TPZ) is a clinically promising anticancer agent that selectively kills the hypoxic cells (*1*, *2*) found in solid tumors (*3*, *4*). Inside cells, one-electron enzymatic reduction of TPZ (Scheme 1) yields an activated form of the drug, **2** (*5*−*7*). In normally oxygenated cells, the radical anion **2a** is rapidly destroyed by reaction with O_2 (*5*, *8*). Although this reaction generates superoxide radical, the toxicity of this species is diminished by cellular enzyme systems such as superoxide dismutase, catalase, and glutathione peroxidase (*9*), and the back-oxidation process represents a detoxification pathway for the activated drug. On the other hand, under the oxygen-poor (hypoxic) conditions found in tumor cells, activated TPZ can partition forward to cause cytotoxic DNA damage (*5*, *10*−*13*).

There is general agreement that one-electron reduction of tirapazamine leads to production of a highly oxidizing DNA-damaging radical; however, the exact identity of this species remains a matter of debate. It has long been proposed that **2b** causes DNA strand breaks via direct abstraction of hydrogen atoms from the deoxyribose

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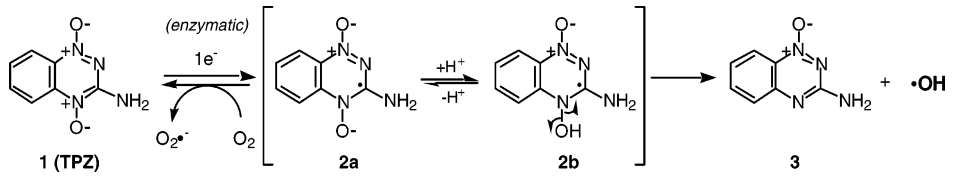
Scheme 1



backbone (*3*). More recently, it has been suggested that **2b** undergoes dehydration to yield a benzotriazinyl radical that is responsible for DNA damage (*14*). Finally, consideration of the sequence specificity of strand cleavage, the spectrum of oxidative base damage caused by activated TPZ, and the results of neutralization-reionization mass spectroscopy experiments has led us to support a mechanism in which **2b** fragments to release the well-known DNA-damaging agent hydroxyl radical as shown in Scheme 1 (*10*, *15*, *16*).

Interestingly, Monge and co-workers in collaboration with researchers at Zeneca Pharmaceuticals reported that the compound 3-amino-2-quinoxalinecarbonitrile 1,4-dioxide (**4**) displays hypoxia-selective cytotoxicity in cell culture comparable to that seen for TPZ (*17*, *18*). The structural similarities between TPZ and **4** are clear, but the ability of **4** to cause DNA damage has not previously been examined. Therefore, we undertook experiments designed to determine whether **4** can cause reductively activated DNA damage under hypoxic conditions. Here, we report that **4** is indeed a redox-activated DNA-damaging agent whose properties are very similar to the clinically promising antitumor agent TPZ. The results reported here shed light on the chemical reactions underlying the medicinally interesting properties of **4** and, more generally, expand the structural range of heterocyclic *N*-oxides known to serve as effective DNA-damaging agents under physiological conditions.

Experimental Section

Materials. Materials were obtained from the following suppliers and were of the highest purity available: xanthine, sodium phosphate, DMSO, mannitol, Aldrich Chemical Co. (Milwaukee, WI); sodium acetate, *N*,*N*′-methylenebisacrylamide, NADPH, desferal, and cytochrome P450 reductase, Sigma Chemical Co. (St. Louis, MO); HPLC grade solvents (acetonitrile, methanol, ethanol, *tert*-butyl alcohol), ethyl acetate, hexane, and acetic acid, Fisher (Pittsburgh, PA); xanthine oxidase, catalase, SOD, acrylamide, and ethidium bromide, Roche Molecular Biochemicals (Indianapolis, IN); Seakem ME agarose, FMC; ethanol, McCormick Distillation Co. (Brookfield, CT); urea, xylene cyanol, bromophenol blue, United States Biochemicals (Cleveland, OH); T4 polynucleotide kinase, New England Biolabs (Beverlym MA); FPG, Endo III, Endo IV, and Exo III, Trevigen (Gaithersburg, MD); 5′-[γ-^{32}P]-dATP, Perkin-Elmer Life Sciences (Boston, MA). Compound **4** was prepared as described previously (*17*, *19*).

Strand Cleavage and Base Damage in Supercoiled Plasmid DNA. In a typical assay, supercoiled plasmid DNA (750 ng) was incubated with *N*-oxide (**4** or **1**, 100−500 μM), NADPH (500 μM), cytochrome P450 reductase (1 mU), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM) in a total volume of 30 μL. Some assays utilized xanthine (500 μM) and xanthine oxidase (12 milliunits, mU) in place of NADPH and cytochrome P450 reductase. Individual components of the DNA-cleavage reactions, except DNA, NADPH, and the enzymes, were degassed prior to use by three freeze−pump−thaw cycles in Pyrex tubes and then torch-sealed under high vacuum. Sealed tubes were scored, transferred to an argon-filled glovebag, opened, and used to prepare individual reactions. Enzymes, NADPH, and DNA were diluted with degassed water in the glovebag to prepare working stock solutions. Reactions were initiated by adding cytochrome P450 reductase, then wrapped with aluminum foil to prevent exposure to light, and incubated for 3 h in the glovebag at room temperature (24 °C). Following incubation, the reactions were stopped by adding 3 μL of 50% glycerol loading buffer, and the resulting mixture was loaded onto a 0.9% agarose gel. The gel was electrophoresed for approximately 3 h at 80 V in 1× TAE buffer and then stained in a solution of aqueous ethidium bromide (0.3 μg/mL) for 1−2 h. DNA in the gel was visualized by UV-transillumination, and the amount of DNA in each band was quantified using an Alpha Innotech IS-1000 digital imaging system. The values reported are not corrected for differential staining of form I and form II DNA by ethidium bromide (*20*). DNA-cleavage assays containing radical scavengers were performed as described above with the exception that radical scavengers such as methanol, ethanol, *tert*-butyl alcohol, DMSO, or mannitol (500 mM) were added to the reaction mixture before addition of cytochrome P450 reductase. For the DNA base damage assays, plasmid DNA (pGL2 basic, 1 μg) was damaged by **4** (50 μM) as described above, then ethanol precipitated and air-dried before being treated with repair enzymes for 16 h at 37 °C, as previously described by our group (*21*). Following incubation, DNA in the assay mixtures was analyzed by agarose gel electrophoresis as described above. Although all of the reactions described above were conducted under hypoxic conditions, there is potential for conversion of adventitious traces of molecular oxygen to superoxide radical in these experiments as shown in Scheme 1. To prevent background DNA damage stemming from the production of superoxide radical, we add superoxide dismutase, catalase, and desferal to our reaction mixtures (*22*). It is clear that these additives efficiently quench superoxide-mediated DNA damage, as background strand cleavage is not observed even under aerobic conditions where superoxide-generating redox cycling of the drug could occur (lane 9, Figure 1).

Sequence Specificity of DNA Cleavage by 3-Amino 2-quinoxalinecarbonitrile 1,4-Di-*N*-oxide (4). A 30-mer 2′-deoxyoligonucleotide with a sequence of (5′-GTC ACGTGCTG-CAGACGACGTGCTGAGCCT) was 5′-end labeled with ^{32}P using T4 polynucleotide kinase and γ-^{32}P ATP (*23*). The labeled oligonucleotide was then annealed with its complimentary strand by heating the mixture to 90 °C followed by slow cooling. Here, we used a system that we described previously for inflicting controlled levels of *N*-oxide-induced damage on duplex DNA (*16*). In this experimental design, DNA and the activating enzyme, cytochrome P450 reductase, were placed inside a dialysis bag and suspended in a buffered solution containing the enzyme substrate and **4**. All reactions were conducted in deoxygenated aqueous buffer inside an inert atmosphere glovebox. In these reactions, drug activation is confined to the relatively small volume inside the dialysis membrane, and the damaged DNA in the dialysis apparatus is easily recovered at the end of the procedure. The substrates and products of the enzymatic drug-activation reaction are free to diffuse in and out of the dialysis bag containing the enzyme−DNA mixture. In this manner, it is possible to maintain moderate, steady concentrations of **4** and NADPH inside the dialysis compartment for extended periods of time, thus allowing the enzymatic activation of **4** to proceed efficiently while avoiding product inhibition and scavenging of drug-derived radicals by the enzyme substrate, which sometimes limit the yields of DNA damage that can be achieved for bioreductively activated drugs in vitro. Thus, a

degassed reaction mixture containing the 30 base pair oligonucleotide duplex (250 000 cpm), **2** (250 μM), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), cytochrome P450 reductase (5 mU), sodium phosphate buffer (10 mM, pH 7.0), and desferal (1 mM) in a total volume of 100 μL was pipetted into a Slide-A-Lyzer minimal unit (Pierce, 3000 MW cutoff). The dialysis unit was then placed with a floater into 2 mL of solution containing **4** (250 μM), NADPH (1 mM), sodium phosphate buffer (10 mM, pH 7.0), and desferal (1 mM) and gently stirred for 15 h in a glovebag filled with argon. The DNA was then removed from the dialysis unit, ethanol precipitated, washed with 70% cold ethanol, and air-dried before dissolving in formamide loading buffer. Finally, the samples were heated for 5 min at 90 °C, cooled in ice, and loaded onto a 16% denaturing polyacrylamide gel. The gel was electrophoresed for 3 h at 1200 V in 1× TBE buffer. Radioactivity on the gel was visualized using a phosphorimager. The Maxam−Gilbert A + G and G-reactions were performed following standard protocols (*24*). DNA cleavage by iron-EDTA/H_2O_2/ascorbate was carried out as described by Tullius and co-workers (*25*).

Characterization of Products Arising from in Vitro Metabolism of 3-Amino 2-Quinoxalinecarbonitrile 1,4-Di-*N*-oxide (4). In a typical assay, a solution of **4** (500 μM), desferal (1 mM), and xanthine or NADPH (1 mM) in sodium phosphate buffer was degassed by three freeze−pump−thaw cycles and then torch-sealed under vacuum. The sealed tube was scored before being transferred to an argon-filled glovebag. The tube was then opened and catalase (100 μg/mL), superoxide dismutase (10 μg/mL), and xanthine oxidase (0.4 U/mL) or cytochrome P450 reductase (0.05 U/mL) were added. The samples were incubated for 3 h in the glovebag at room temperature. After incubation, proteins and DNA were removed by centrifugation through Amicon Microcon (YM3) filters. The filtrate was analyzed by HPLC employing a C18 reverse phase Rainin Microsorb-MV column (5 μm particle size, 100 Å pore size, 25 cm length, 4.6 mm i.d.) eluted with a isocratic mobile phase of 30% methanol and 70% water with 0.5% acetic acid at a flow rate of 0.8 mL/min. The products were monitored by UV absorbance at 254 nm. In vitro metabolism by NADPH:cytochrome P450 reductase yields two major products whose relative yields were estimated on the basis of their relative peak areas in the HPLC chromatogram. These products were identified as mono-*N*-oxide analogues of **4** using LC/MS and LC/MS/MS (see Supporting Information). The major metabolite was prepared on a larger scale as described below and was determined to be the 4-oxide (**6**) based upon its ^{1}H NMR, ^{13}C NMR, and, ultimately, X-ray crystallography (see Supporting Information). When the in vitro metabolism of **4** was performed in the presence of 2-mercaptoethanol (5 mM), a new product was observed. This product was prepared on a larger scale as described below, and its structure was determined on the basis of ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. The identity of the major product resulting from the reduction of **4** by NADPH:cytochrome P450 reductase was confirmed by co-injection with an authentic sample.

Large-Scale, Enzymatic Synthesis of 3-Amino 2-Quinoxalinecarbonitrile-4-*N*-oxide (6). Compound **6** was prepared on a large scale by xanthine/xanthine oxidase-mediated reduction of **4**. To an aqueous solution of **4** (17 mg, 0.08 mmol) in a 50 mM sodium phosphate buffer, pH 7.0, containing 20% DMSO, were added xanthine (30 mg, 0.2 mmol) and xanthine oxidase (50 mU). The resulting reaction mixture was incubated for 5 h, followed by addition of another 50 mU of xanthine oxidase and incubation for another 5 h. The solution was extracted with ethyl acetate (3×), the organic layers were combined and dried over anhydrous sodium sulfate, and the solvent was removed under vacuum by rotary evaporation with warming. The resulting yellow solid was purified by column chromatography on silica gel (eluted with ethyl acetate) to yield 10.3 mg (66%) of **6**: R_f = 0.6 (ethyl acetate), ^{1}H NMR (DMSO-d_6) δ 8.25 (d, J = 8.7 Hz, 1H), 8.0 (d, J = 8.5 Hz, 1H), 7.99 (br s, 2H), 7.87 (t, J = 7.8 Hz, 1H), 7.66 (t, J = 7.7 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 145.9, 137.3, 136.4, 133.6, 130.5, 127.9, 118.7, 117, 114.9. This material is identical to that obtained by the synthetic route described for this compound by Monge et al. (*26*). Mass spectrometric and X-ray crystallographic characterization of this compound are provided in the Supporting Information.

Large-Scale, Enzymatic Synthesis of 3-Amino 2-Quinoxalinecarbamide-4-*N*-oxide (8). Compound **8** was prepared by utilizing the same procedure used for **6** with the exception that the reaction contained 100 mM 2-mercaptoethanol. The products were extracted with ethyl acetate, and the solvent was removed by rotary evaporation with warming. The resulting yellow solid was purified by column chromatography on silica gel (eluted with ethyl acetate) to yield 9.4 mg (56%) of **8**: R_f = 0.27 (ethyl acetate), ^{1}H NMR (DMSO-d_6, containing a drop of D_2O) δ 8.25 (d, J = 9.7 Hz, 1H), 7.99 (d, J = 9.3 Hz 1H), 7.81 (t, J = 8.4 Hz, 1H), 7.64 (t, J = 8.3 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 167.5, 144.2, 135.7, 135, 132.6, 132.4, 130.4, 127.4, 116.9. Information regarding the mass spectrometric characterization of this compound is provided in the Supporting Information.

Rate of Reduction of 3-Amino 2-Quinoxalinecarbonitrile-1,4-di-*N*-oxide (4) and Tirapazamine (1) by NADPH: Cytochrome P450 Reductase. A solution of **4** or **1** (250 μM), desferal (1 mM), and NADPH (500 μM) in sodium phosphate buffer (50 mM, pH 7.0) was deoxygenated by three cycles of freeze−pump−thaw degassing and torch-sealed under vacuum before being transferred into an argon-filled glovebag. The sealed tube was then opened, the solution was transferred to a quartz cuvette, and the cuvette was sealed with a septum. The cuvette was then placed into the UV−vis instrument, and NADPH:cytochrome P450 reductase (50 mU) was injected through the septum to initiate the reaction. The rate of reduction was followed by monitoring the disappearance of the di-*N*-oxide absorbance maxima in the UV−vis spectrum (480 nm for **4** and 474 nm for **1**) over a period of 5000 s.

Results

Hypoxia-Selective, Enzyme-Activated DNA Damage by 4. Early studies showed that **4** is cytotoxic against V79 cells under hypoxic conditions (*17*, *18*). Motivated by the structural similarities between **4** and TPZ, we set out to determine whether this quinoxaline di-*N*-oxide causes redox-activated DNA damage under hypoxic conditions, in a manner similar to that observed for the triazine *N*-oxide, TPZ.

In these studies, we used either NADPH:cytochrome P450 reductase or xanthine/xanthine oxidase for reductive activation of **4**. These enzymes were chosen because NADPH:cytochrome P450 reductase is thought to be responsible for the in vivo activation of TPZ (*11*, *27*, *28*) and xanthine/xanthine oxidase has been used successfully for the activation of TPZ in a number of in vitro studies (*5*, *10*, *16*, *29*). For reactions carried out under hypoxic conditions, molecular oxygen was removed from the solutions by freeze−pump−thaw degassing and the assay mixtures were prepared in an inert atmosphere glovebag.

We initially examined the ability of **4** to damage supercoiled plasmid DNA. In this assay, DNA strand cleavage causes conversion of supercoiled (form I) plasmid DNA to the open-circular form (form II). Yields of DNA strand cleavage can easily be quantified using agarose gel electrophoresis. We find that compound **4** causes DNA strand cleavage when incubated with the NADPH:cytochrome P450 enzyme system under hypoxic conditions (Figure 1). The yields of DNA cleavage by **4** are comparable to those for TPZ (Figure 2). When **4** is incubated with the enzyme alone, NADPH alone (data not shown), or under aerobic conditions, no DNA strand

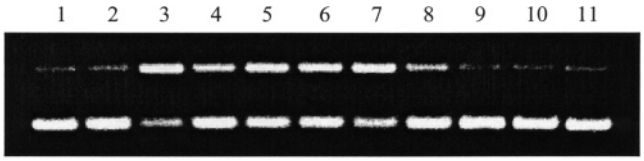


Figure 1. Cleavage of supercoiled plasmid DNA by **4** in the presence of NADPH:cytochrome P450 reductase as an activating system. All reactions contained DNA (750 ng, pGL-2 Basic), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), and desferal (1 mM) and were incubated under anaerobic conditions (except lane 9) at 24 °C for 16 h, followed by agarose gel electrophoretic analysis. Lane 1, DNA alone ($S = 0.23 \pm 0.04$); lane 2, NADPH (500 μM) + reductase (1 mU) ($S = 0.25 \pm 0.03$); lane 3, **4** (500 μM) + NADPH (500 μM) + reductase ($S = 1.21 \pm 0.17$); lanes 4−8, **4** (500 μM) + NADPH (500 μM) + reductase + methanol (500 mM, lane 4) ($S = 0.6 \pm 0.22$); ethanol (500 mM, lane 5) ($S = 0.59 \pm 0.06$); *tert*-butyl alcohol (500 mM, lane 6) ($S = 0.45 \pm 0.11$); DMSO (500 mM, lane 7) ($S = 0.59 \pm 0.24$); mannitol (500 mM, lane 8) ($S = 0.30 \pm 0.04$); lane 9, **4** (500 μM) + NADPH (500 μM) + reductase + air ($S = 0.23 \pm 0.05$); lane 10, **6** (500 μM) + NADPH (500 μM) + reductase ($S = 0.21 \pm 0.03$); lane 11, **4** alone ($S = 0.23 \pm 0.01$). The value S represents the mean number of strand breaks per plasmid molecule and is calculated using the equation $S = -\ln f_1$, where f_1 is the fraction of plasmid present as form I.

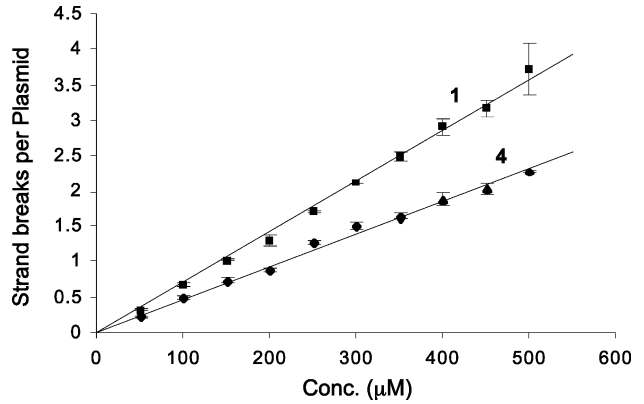


Figure 2. Comparison of anaerobic DNA-cleavage efficiency by various concentrations of **1** or **4** in the presence of NADPH: cytochrome P450 reductase as an activating system. Supercoiled plasmid DNA (750 ng) was incubated with **4** or **1** (100−500 μM), NADPH (500 μM), cytochrome P450 reductase (1 mU), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM) under anaerobic conditions at room temperature for 3 h, followed by agarose gel electrophoretic analysis. Strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$, where f_1 is the fraction of plasmid present as form I.

cleavage is observed. Compound **6**, the major metabolite produced by the reductive metabolism of **4** (see below) does not produce DNA-strand cleavage either alone (data not shown) or in the presence of NADPH:cytochrome P450 reductase under hypoxic conditions. Redox-activated DNA cleavage by **4** is inhibited by classical radical-scavenging agents (*22*) such as ethanol, methanol, *tert*-butyl alcohol, DMSO, and mannitol.

Interestingly, incubation of compound **4** with the xanthine/xanthine oxidase enzyme system under hypoxic conditions does not lead to DNA strand cleavage (data not shown). This was somewhat surprising, given that this enzyme system efficiently triggers DNA damage by TPZ (*10*). Results of the in vitro metabolism studies described in subsequent sections of this paper ultimately provided an explanation for this phenomenon.

In a separate assay, we investigated whether reductive activation of **4** by NADPH:cytochrome P450 reductase

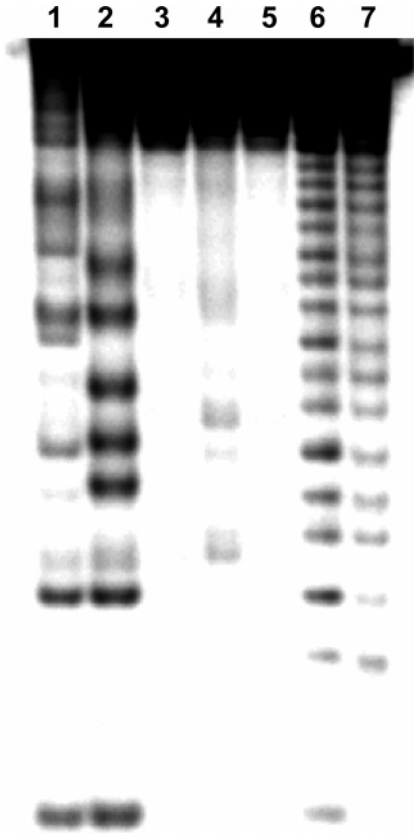
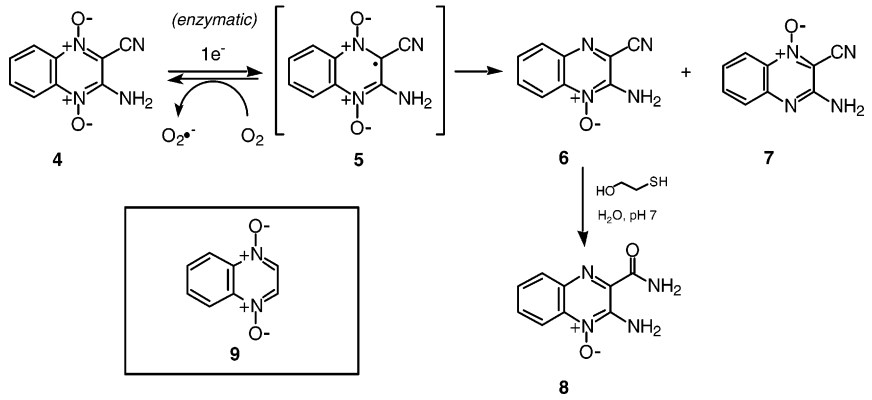


Figure 3. Cleavage of a 5′-^{32}P labeled 30-mer oligonucleotide duplex by enzyme-activated **4**. All reactions contained 5′-^{32}P labeled 30 mer oligonucleotide duplex (250 000 cpm), sodium phosphate buffer (10 mM, pH 7.0), desferal (1 mM), SOD (10 μg/mL), and catalase (100 μg/mL). The reactions were incubated for 16 h under anaerobic conditions at 24 °C, followed by 20% polyacrylamide gel electrophoresis. Lane 1, G-reaction; lane 2, A + G reaction; lane 3, DNA alone; lane 4, DNA + NADPH + cytochrome P450 reductase; lane 5, DNA + **4** only; lane 6, DNA + **4** + NADPH + cytochrome P450 reductase; lane 7, DNA + iron-EDTA/H_2O_2/ascorbate.

under hypoxic conditions leads to DNA base damage in addition to the direct strand cleavage described above. Following exposure of the DNA to activated **4** under hypoxic conditions, base damage was revealed by treating the plasmid with the base excision repair enzyme endo III (for a review of the use of repair endonucleases to study DNA damage, see refs *21*, *30*). This enzyme excises a variety of oxidized pyrimidine and purine lesions from duplex DNA and catalyzes strand cleavage at the resulting abasic site (*31*−*33*). We find that treatment of the damaged DNA with endo III does indeed cause an increase in strand breaks. A control experiment shows that treatment of the damaged DNA with the enzyme endo IV, which catalyzes strand cleavage at abasic sites and oxidized abasic sites (*30*), also results in an increase in the number of strand breaks. Importantly, however, endo III treatment reveals 2.0 ± 0.1 times more strand breaks than the endo IV control reaction. Together these findings provide evidence that the strand cleavage resulting from endo III treatment is due primarily to excision of oxidized base lesions and not the enzyme's ability to cause cleavage at abasic sites. Similar findings have been reported previously for DNA damage by activated TPZ (*21*).

Finally, we examined cleavage of a ^{32}P-labeled oligonucleotide duplex by activated **4** under hypoxic conditions (Figure 3). We find that DNA strand cleavage occurs at

Scheme 2



every base pair in the duplex, with nearly complete lack of sequence specificity. The pattern of DNA strand cleavage caused by **4** is essentially identical to that caused by iron-EDTA and is characteristic of a highly oxidizing, radical species such as hydroxyl radical (see ref *25*). Previous studies showed that reductively activated TPZ cleaves duplex DNA with a similar lack of sequence selectivity (*10*).

Products Resulting from in Vitro Metabolism of 4 by NADPH:Cytochrome P450 Reductase and Xanthine Oxidase. We find that in vitro metabolism of **4** by NADPH:cytochrome P450 reductase under anaerobic conditions yields the 4-oxide, **6** (Scheme 2), as the major product, along with smaller amounts of the 1-oxide, **7** (approximately a 7:1 mixture of **6**:**7**). Under the conditions reported here, the yields of **6** and **7** are approximately 75% and 10%, respectively, based upon starting di-*N*-oxide (**4**). The production of **6** (rather than **7**) as the major metabolite stemming from reductive activation of **4** is consistent with a chemical mechanism analogous to that shown for TPZ in Scheme 1. The cyano group is superior to the amine group in its radical-stabilizing properties (*34*), likely leading to increased radical spin density at C-2 in the reduced quinoxaline (as depicted in structure **5** of Scheme 2). In turn, increased spin density at C-2 is expected to favor loss of the 1-oxide oxygen to produce **6**. The conversion of **4** to these mono-*N*-oxide metabolites, like DNA damage by the compound, is completely inhibited under aerobic conditions, consistent with the idea that both processes proceed via the same oxygen-sensitive intermediate (**5**, Scheme 2). The major metabolite, **6**, was characterized by ^{1}H NMR, ^{13}C NMR, electrospray mass spectroscopy, comparison to authentic synthetic standards, and, ultimately, by X-ray crystallography (see Supporting Information). The minor metabolite (**7**) was characterized by LC/MS and LC/MS/MS. UV−vis spectroscopy was used to monitor the consumption of TPZ and **4** by NADPH:cytochrome P450 reductase under anaerobic conditions. These assays showed that compound **4** and TPZ are metabolized at similar rates under the reaction conditions employed for these studies (Figure 4).

When the in vitro metabolism experiments were conducted in the presence of 2-mercaptoethanol (5 mM), we observed formation of a new metabolite. This compound was characterized as the hydrolysis product **8** using ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. We recognized that this product could arise via a known reaction involving thiol-catalyzed hydrolysis of the nitrile group (*35*, *36*). In principle, hydrolysis of the nitrile group could

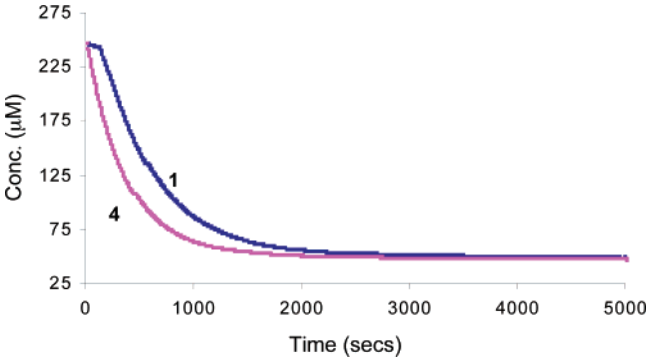


Figure 4. Rate of reduction of **1** (250 μM) or **4** (250 μM) by NADPH:cytochrome P450 reductase under anaerobic conditions measured by UV−vis spectroscopy. A solution of **1** (250 μM) or **4** (250 μM), NADPH (500 μM), and desferal (1 mM) in sodium phosphate buffer (50 mM, pH 7.0) was incubated with reductase (50 mU) under anaerobic conditions at 24 °C. The rate of reduction was monitored by measuring the disappearance of the di-*N*-oxide absorbance at 474 nm (**1**) or 480 nm (**4**) over time.

occur on either the starting material (**4**) or the mono-*N*-oxide metabolite **6**. In practice, we find that incubation of the metabolite **6** with 2-mercaptoethanol leads to production of **8** (with a half-life of 1.1 h under the reaction conditions employed here, 5 mM 2-mercaptoethanol, pH 7). The corresponding reaction in the presence of the biological thiol glutathione (5 mM) is relatively slow, yielding only ~7% conversion to hydrolysis product over the course of 10 h. The nitrile groups in the di-*N*-oxide **4** starting material and the minor mono-*N*-oxide metabolite **7** do not undergo significant thiol-catalyzed hydrolysis when treated with 2-mercaptoethanol (5 mM).

We also investigated the in vitro metabolism of **4** by the xanthine/xanthine oxidase enzyme system. Incubation of **4** with this enzyme system under hypoxic conditions leads to efficient conversion of **4** to the mono-*N*-oxide **6**. Thus, xanthine oxidase effectively carries out reductive metabolism of **4**, yet, as described above, does not activate the DNA-damaging properties of the compound. Insight into this apparent contradiction was provided by the observation that the conversion of **4** to **6** mediated by xanthine oxidase proceeds smoothly even under aerobic conditions. This indicates that the xanthine oxidase-mediated reduction process does not occur via an oxygen-sensitive radical intermediate such as **5** (Scheme 2). Rather, it appears that xanthine oxidase mediates the direct two-electron reduction of **4** to **6** without release of activated drug (**5**) from the active site of the enzyme. It is known that xanthine oxidase has the capacity to carry out either one-electron or two-electron reduction of vari-

ous substrates (*37*); however, the inability of xanthine oxidase to convert **4** to the one-electron reduced intermediate **5** is noteworthy in light of the fact that this enzyme efficiently mediates one-electron reductive activation of TPZ (*5*, *10*). The metabolism of **4** by xanthine oxidase reported here is analogous to DT-diaphorase-mediated conversion of TPZ to its mono-*N*-oxide **3**, which also occurs by direct two-electron reduction and, therefore, is not inhibited by molecular oxygen (*38*).

Conclusions

Our studies provide the first evidence that the hypoxia-selective cytotoxin **4** is able to cause redox-activated DNA damage under hypoxic conditions. The results provide a clear chemical basis for the medicinally interesting biological activities reported for this compound (*17*, *18*, *39*−*42*). The findings indicate that one-electron reduction of **4** by NADPH:cytochrome P450 reductase yields an oxygen-sensitive intermediate (**5**) that can partition forward to cause DNA damage under low-oxygen conditions. Overall, it is clear that reductive activation of **4** leads to production of a highly oxidizing, freely diffusible radical that causes both sequence-independent strand breaks via abstraction of hydrogen atoms from the deoxyribose backbone and oxidative damage to the heterocyclic bases of DNA. On the basis of the experiments reported here, the nature of the DNA damage caused by reductively activated **4** is completely analogous to that seen for the drug TPZ (*10*, *16*, *21*). Thus, the work provides a chemical foundation for an expectation that the biological properties of **4** may be analogous to those of the more extensively studied drug TPZ.

Previous studies demonstrated that the unsubstituted quinoxaline di-*N*-oxide (**9**) is a relatively poor hypoxia-selective DNA-damaging agent as compared to TPZ (*43*). In contrast, the yields of DNA damage by the substituted quinoxaline di-*N*-oxide **4** studied here are comparable to those seen for TPZ. Together, these observations may serve to emphasize the important role that reduction potential plays in determining the DNA-damaging properties of heterocyclic *N*-oxides. It has previously been noted that enzyme-activated, hypoxia-selective cytotoxins should have reduction potentials in the range of −0.1 to −0.5 V (vs NHE in pH 7 water) (*44*, *45*). Compounds with reduction potentials below −0.5 V are not efficiently reduced by cellular enzymes, while compounds with reduction potentials above −0.1 V are easily reduced, but yield relatively stable intermediates that are not sensitive to O_2 (*44*). It is likely that **9**, with a half-wave potential of −1.21 V (vs SCE in DMF) (*46*), is a relatively inefficient DNA-damaging agent because it is not effectively reduced by relevant reductases. However, addition of cyano and amino substituents to the quinoxaline di-*N*-oxide core yields a compound (**4**) with a reduction potential nearly identical (*17*, *18*) to that of the clinically promising antitumor agent TPZ (−0.45 V) (*47*). In turn, we find that the chemical mechanism and efficiency of DNA damage by this substituted quinoxaline di-*N*-oxide **4** closely resembles that of TPZ. Compound **4** is the first heterocyclic *N*-oxide demonstrated to possess reductively activated DNA-damaging properties comparable to those of TPZ. In general, these findings highlight the capacity for structurally diverse heterocyclic *N*-oxides with appropriate reduction potentials to cause redox-activated DNA damage inside cells.

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Supporting Information Available: Hydrolysis of **6**, HPLC traces of **4**, ESI LC-MS and LC-MS/MS analysis, synthesis of materials, and crystal structure and structural data. This material is available free of charge via the Internet at http://pubs.acs.org.

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