Reaction of the Hypoxia-Selective Antitumor Agent Tirapazamine with a C1′-Radical in Single-Stranded and Double-Stranded DNA: The Drug and Its Metabolites Can Serve as Surrogates for Molecular Oxygen in Radical-Mediated DNA Damage Reactions†

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Received June 29, 1999; Revised Manuscript Received August 25, 1999

ABSTRACT: The compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (**1**, tirapazamine; also known as SR4233, WIN 59075, and tirazone) is a clinically promising anticancer agent that selectively kills the oxygen-poor (hypoxic) cells found in tumors. When activated by one-electron enzymatic reduction, tirapazamine induces radical-mediated oxidative DNA strand cleavage. Using the ability to generate a single deoxyribose radical at a defined site in an oligonucleotide, we recently provided direct evidence that, in addition to *initiating* the formation of DNA radicals, tirapazamine can *react with* these radicals and convert them into base-labile lesions [Daniels et al. (1998) *Chem. Res. Toxicol. 11*, 1254−1257]. The rate constant for trapping of a C1′-radical in single-stranded DNA by tirapazamine was shown to be $\sim 2 \times 10^8\ M^{-1}\ s^{-1}$, demonstrating that tirapazamine can substitute for molecular oxygen in radical-mediated DNA strand damage reactions. Because reactions of tirapazamine with DNA radicals may play an important role in its ability to damage DNA, we have further characterized the ability of the drug and its metabolites to convert a C1′-DNA radical into a base-labile lesion. We find that tirapazamine reacts with a C1′-radical in double-stranded DNA with a rate constant of $4.6 \times 10^6\ M^{-1}\ s^{-1}$. The mono-*N*-oxide (**3**) stemming from bioreductive metabolism of tirapazamine converts the C1′-radical to an alkaline-labile lesion more effectively than the parent drug. Compound **3** traps a C1′-radical in single-stranded DNA with a rate constant of $4.6 \times 10^8\ M^{-1}\ s^{-1}$ and in double-stranded DNA with a rate constant of $1.4 \times 10^7\ M^{-1}\ s^{-1}$. We have also examined the rate and mechanism of reactions between the C1′-radical and representatives from two known classes of "oxygen mimetic" agents: the nitroxyl radical 2,2,6,6-tetramethylpiperidin-*N*-oxyl (**4**, TEMPO) and the nitroimidazole misonidazole (**5**). TEMPO traps the C1′-radical in single-stranded DNA ($7.2 \times 10^7\ M^{-1}\ s^{-1}$) approximately 3 times less effectively than tirapazamine, but 2 times as fast in double-stranded DNA ($9.1 \times 10^6\ M^{-1}\ s^{-1}$). Misonidazole traps the radical in single- ($6.9 \times 10^8\ M^{-1}\ s^{-1}$) and double-stranded DNA ($2.9 \times 10^7\ M^{-1}\ s^{-1}$) with rate constants that are roughly comparable to those measured for the mono-*N*-oxide metabolite of tirapazamine. Finally, information regarding the chemical mechanism by which these compounds oxidize a monomeric C1′-nucleoside radical has been provided by product analysis and isotopic labeling studies.

In cancer chemotherapy, there has been a longstanding desire to identify features unique to cancer cells that might be used to target the action of chemotherapeutic agents specifically toward these unwanted cells. This is a difficult task because in many ways cancer cells closely resemble normal cells. It is now well established, however, that many solid tumors contain significant regions that are poorly oxygenated (hypoxic) (*1, 2*). Thus, hypoxia is a unique physical feature of tumor cells that can potentially be exploited in cancer chemotherapy (*3*−*5*).

The compound 3-amino-1,2,4-benzotriazine 1,4-*N*-oxide (**1**, tirapazamine; also known as SR4233, WIN 59075, and tirazone) displays remarkable selective toxicity toward oxygen-poor cells (*3, 6, 7*). In experiments utilizing human and rodent tumor cell lines, tirapazamine is 15−200 times more toxic to hypoxic cells than it is toward normally oxygenated cells. Accordingly, tirapazamine shows promising antitumor properties and is currently undergoing a variety of phase I, II, and III trials for the treatment of head and neck cancers, glioblastoma, and non-small-cell lung cancer (*6*). Tirapazamine is generally used in combination with radiation or cisplatin (*6, 8*−*10*).

† This work was supported by the NIH (GM-54996) and Alfred P. Sloan Foundation (M.M.G.) and by the University of Missouri Research Board (K.S.G.).

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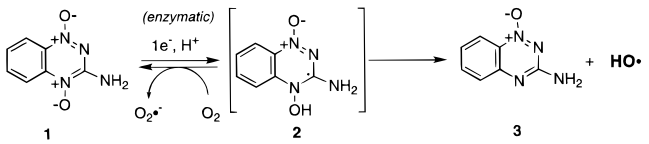
10.1021/bi991488n CCC: $18.00
Published on Web 10/05/1999

Scheme 1

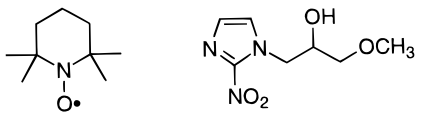


Tirapazamine derives its biological activity by causing DNA strand cleavage (*7*, *11*, *12*). DNA cleavage is initiated by one-electron enzymatic reduction of the drug to yield the activated intermediate **2** (Scheme 1) (*3*, *13*−*15*). The exact nature of the drug-derived species responsible for DNA strand scission remains under investigation. It is often suggested (*6*) that the drug radical **2** directly abstracts hydrogen atoms from DNA; however, preliminary evidence has been presented (*13*) indicating that the activated drug (**2**) breaks down to release the well-known DNA-damaging agent hydroxyl radical (Scheme 1). Tirapazamine's specific action against oxygen-poor cells stems from the fact that the activated form of the drug (**2**) is rapidly deactivated by reaction with O_2 to regenerate **1** and superoxide radical (Scheme 1).

The scenario for hypoxia-selective DNA cleavage by tirapazamine described above presents something of a paradox: on the one hand, significant levels of the drug-derived DNA-cleaving radical are produced only under *low-oxygen* conditions, while on the other hand, it is well-known that O_2 enhances radical-mediated DNA strand cleavage in vivo (*16*, *17*). This oxygen effect on radical-mediated DNA strand cleavage in biological systems reflects the ability of O_2 to compete effectively with endogenous thiols for reaction with DNA radicals (*18*−*21*). Reaction of O_2 with deoxyribose radicals in DNA (an event sometimes referred to as "fixing" of the radical lesion) generally leads to DNA strand cleavage, (*22*−*25*) whereas the reaction with a thiol generally represents a chemical repair process (*18*, *21*).

Recent reports have shed light on chemical properties that may allow tirapazamine to efficiently cause DNA strand cleavage under hypoxic conditions. We (*26*) and others (*27*) have shown that, in addition to *initiating* the formation of DNA radicals, tirapazamine can *react with* these radicals and convert them into base-labile lesions. Tirapazamine traps the C1′-radical in single-stranded DNA with a rate constant of $\sim 2 \times 10^8\ M^{-1}\ s^{-1}$ (*26*). Thus, our recent work provided the first direct evidence that, under low-oxygen conditions, tirapazamine can substitute for molecular oxygen in radical-mediated DNA strand damage reactions.

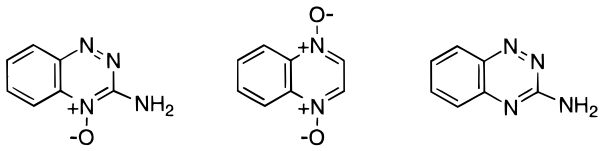
Because the interaction of tirapazamine with DNA radicals may play an important role in its ability to cleave DNA efficiently under low-oxygen conditions, we have further characterized the chemical reaction between this drug and the C1′-DNA radical. Using the ability to generate a C1′-radical at a defined site in DNA, we have measured rate constants for the reaction of tirapazamine and its metabolites with the radical in single- and double-stranded DNA. In addition, we report isotopic labeling studies and product analysis that provide insight regarding the chemical mechanism by which these compounds oxidize the C1′-radical. Finally, we have compared the reactivity of tirapazamine to those of representatives from two classes of known "oxygen mimetic" agents: the nitroxyl radical 2,2,6,6-tetramethylpiperidin-*N*-oxyl (TEMPO,[1] **4**) and the nitroimidazole misonidazole (**5**).



4 **5**

EXPERIMENTAL SECTION

General Procedures. Oligonucleotide synthesis was carried out on an Applied Biosystems Incorporated 380B DNA synthesizer using standard protocols as previously described (*18*, *21*). Phosphoramidites and all other oligonucleotide synthesis reagents were obtained from Glen Research. DNA manipulation, including enzymatic labeling, was carried out using standard procedures (*28*). Oligonucleotides were sequenced using a reaction specific for adenine (*29*). Preparative and analytical oligonucleotide separations were carried out on a 20% polyacrylamide denaturing gel [5% cross-link, 45% urea (by weight)]. T4 polynucleotide kinase was obtained from New England Biolabs. [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech. Radioactive samples were counted by Cerenkov counting, using a Packard Tri-Carb 1500 scintillation counter. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager equipped with Imagequant Version 3.3 software. All photolyses of oligonucleotides were carried out in Pyrex tubes (6 mm i.d.) using a Rayonet Photoreactor (RPR-100) equipped with 16 lamps having a maximum output at 350 nm, and a rotating sample holder. The samples were degassed using 3 freeze−pump−thaw cycles and sealed under vacuum. Compounds **1**, **3**, and **18** were prepared by the methods of Mason and Tennant (*30*). Compound **11** was prepared by the method of Sharpless and co-workers (*31*) or by the method of Landquist (*32*). Compound **10** was independently prepared by reaction of *tert*-butylperacetate with **1** or the reaction of m-CPBA with **18** (Fuchs and Gates, manuscript in preparation).



10 **11** **18**

General Procedure for Analysis of Competition between β-Mercaptoethanol and Di-N-oxides. Radiolabeled oligonucleotide (**8** or **9**), in 10 mM phosphate (pH 7.2), 100 mM

5′-d(GTC ACG TGC TGC A**6**A CGA CGT GCT GAG CCT)
8

5′-d(GTC ACG TGC TGC A**6**A CGA CGT GCT GAG CCT)
(CAG TGC ACG ACG TAT GCT GCA CGA CTC GGA)d-5′
9

NaCl, β-mercaptoethanol (0−100 mM), and the appropriate O_2 surrogate (0.25−1 mM) were photolyzed under anaerobic

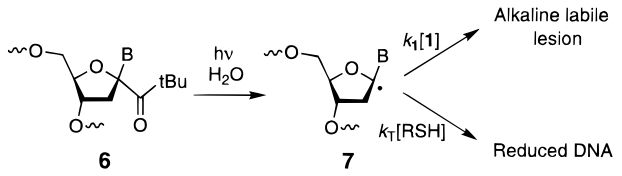
[1] Abbreviations: TEMPO, 2,2,6,6-tetramethylpiperidin-*N*-oxyl; BPB, bromophenol blue; BSTFA, bis(trimethylsilyl)trifluoromethylacetamide; GC/MS, tandem gas chromatography/mass spectrometry; m-CPBA, *meta*-chloroperbenzoic acid.

conditions. In a given experiment, the concentration of thiol was varied, but the concentration of O_2 surrogate was the same in all tubes. Each concentration of β-mercaptoethanol was carried out in duplicate. Five samples were irradiated without β-mercaptoethanol, because the average amount of piperidine cleavage under these conditions was used to determine the amount of trapped product in samples containing β-mercaptoethanol. Single-stranded substrates (**8**) were photolyzed for 20 min. Double-stranded substrates (**9**) were photolyzed for 2 h. The photolyzed solution was transferred into an eppendorf tube, and the photolysis tube was washed with H_2O (2×50 μL). The combined material was ethanol precipitated. The precipitated photolysate was resuspended in piperidine (1.0 M) and heated at 90 °C for 20 min. Following heating, the samples were lyophilized, resuspended in H_2O, and lyophilized again (3×100 μL). Prior to loading on the gels, the DNA pellets were resuspended in formamide loading buffer by vortexing for 3 min, heating at 55 °C for 4 min, and vortexing again for 1 min. Samples in adjacent lanes were loaded in a staggered manner (30 min), so that there would not be any overlap of products, and were analyzed using 20% denaturing polyacrylamide gel electrophoresis. Gels were run until the BPB dye was 1 in. from the bottom of the plate. Quantitative analysis was carried out using a Molecular Dynamics Phosphorimager. The amount of trapped product was determined by subtracting the amount of cleavage in a sample at a given β-mercaptoethanol concentration from the average amount of cleavage in tubes that had no β-mercaptoethanol present. Concentrations of O_2 surrogates used: **1**, **10**, **11**, single- (**8**) and double-stranded (**9**) DNA (1 mM); **3**, single- and double-stranded DNA (0.5 mM); **4**, **5**, single-stranded (0.1 mM), double-stranded (1 mM) DNA.

*General Procedure for Determining Isotopic Incorporation from $H_2{}^{18}O$ in 2′-Deoxyribonolactone (**17**).* The samples containing **12** (0.05 mM), O_2 surrogate (e.g., **1**, 1 mM) in $H_2{}^{18}O$ (50 μL), were sealed as described above, and photolyzed for 30 min in a Rayonet photoreactor. The samples were transferred to conical (1 mL) Kontes vials, and the photolysis tubes were washed with H_2O (30 μL). The samples were lyophilized, and then lyophilized from EtOH (2×50 μL). BSTFA (30 μL) was added; the sample was vortexed (1 min) and heated at 105 °C (1.5 h). The samples were then analyzed by GC/MS (SIM mode; *m*/*z* 261, 263; 120 °C for 2 min, 20 °C/min up to 300 °C, 5 min at 300 °C).

*General Procedure for the Detection of Deoxygenated Products in the Reaction of N-Oxides with 2′-Deoxyuridin-1′-yl (**13**).* In a typical assay (final volume 75 μL), a Pyrex tube (i.d. 3 mm, o.d. 5 mm) containing sodium phosphate buffer (20 mM, pH 7.0), **12** (50 μM), and **1** (50−100 μM) was freeze−pump−thaw degassed (3×) and sealed under vacuum, followed by photolysis (14−18 min) employing a medium-pressure mercury lamp (Conrad Honovia 7825 medium-pressure mercury arc lamp, operating at 450 W, placed in a water-cooled Pyrex immersion well; >300 nm transmitted), at ambient temperature (24−27 °C). The assays were then analyzed by HPLC, employing a C-18 reverse-phase Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.) eluted with an isocratic mobile phase composed of 1% acetic acid, 25% MeOH, and 74% water at a flow rate of 0.9 mL/min. The products were monitored by UV detection (254 nm). The deoxygenated products were identified by comparison of their retention times to those of independently prepared standards and confirmed by co-injection experiments. Some background photolytic decomposition, including some *N*-deoxygenation with longer photolysis times, is observed in control reactions involving photolysis of the *N*-oxide in the absence of the radical precursor **12**. Photolytic deoxygenation is a well-known reaction for *N*-oxides (*33*), and some aspects of the photolytic deoxygenation of **1** have previously been studied (*34*). In all cases, the respective *N*-deoxygenation products were the major new products arising from reaction of the *N*-oxides (**1**, **3**, **10**) with the nucleoside radical **13**.

Scheme 2



RESULTS AND DISCUSSION

Rate Constants for Trapping of a C1′-Radical in Single-Stranded and Double-Stranded DNA by Tirapazamine and Other N-Oxides. Using a photolabile radical precursor (**6**) synthetically incorporated into DNA oligonucleotides, we recently demonstrated that tirapazamine converts a C1′-radical (**7**) in single-stranded DNA to an alkaline-labile lesion with a rate constant of 2.5×10^8 M^{-1} s^{-1} (*26*). Interaction of tirapazamine with the C1′-radical is medicinally relevant because it is likely that C1′-radicals are generated during the course of DNA strand damage mediated by **1** and by γ-radiolysis. This is suggested by the observation that tirapazamine generates hydroxyl radical or a species of similar reactivity (*13*), along with the report that hydroxyl radical reacts with DNA at the C1′-position (*23*).

In these experiments, we ask the biologically relevant question of whether the *N*-oxides under study can compete with thiols for reaction at the DNA radical. The C1′-radical can either react with thiol (to yield chemically repaired, non-alkaline-labile DNA) or react with the *N*-oxide to generate an alkaline-labile lesion that can be observed by gel electrophoresis as a DNA strand break, following piperidine workup (*26*). The yield of strand cleavage versus repair is dictated by the relative rates for trapping of the radical by *N*-oxide and trapping by thiol, respectively (Scheme 2). The rate constant for trapping of the C1′-nucleotide radical by β-mercaptoethanol has been estimated in single- and double-stranded DNA (*21*) ($\sim 4.4 \times 10^6$ and $\sim 1.8 \times 10^6$ M^{-1} s^{-1}, respectively); therefore, determination of the total yield of alkaline-labile lesions and direct strand breaks (labile lesions) obtained in the presence of known concentrations of thiol and *N*-oxide can provide the overall rate constant for reaction of the *N*-oxide with the C1′-radical (eq 1) (*18, 21, 26*):

$$\frac{\text{cleavage}(\mathbf{1})}{\text{trapping(RSH)}} = \frac{k_1[\mathbf{1}]}{k_T} \frac{1}{[\text{RSH}]} \qquad (1)$$

Specifically, in these experiments, samples of the single-stranded or double-stranded oligonucleotide (5′-^{32}P-**8** or 5′-

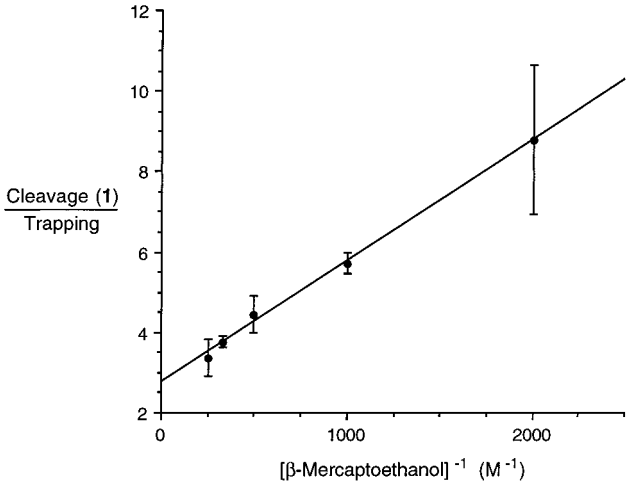


FIGURE 1: Determination of the effect of β-mercaptoethanol on the formation of alkaline-labile lesions from 2′-deoxyuridin-1′-yl (**7**) in double-stranded DNA (**9**) in the presence of tirapazamine (**1**, 1 mM).

Table 1: Rate Constants for the Trapping of a C1′-Radical in Single-Stranded (**8**) and Double-Stranded (**9**) DNA by Various N−O-Containing Compounds

	trapping rate constant $\times 10^{-7}$ (M^{-1} s^{-1})	
compound	**8**	**9**
tirapazamine (**1**)	25.0 ± 2.80	0.46 ± 0.11
3-amino-1,2,4-benzotriazine 1-oxide (**3**)	46.3 ± 14.1	1.41 ± 0.14
3-amino-1,2,4-benzotriazine 4-oxide (**10**)	13.4 ± 0.01	3.10 ± 0.12
quinoxaline 1,4-oxide (**11**)	2.10 ± 0.73	ND
TEMPO (**4**)	7.21 ± 0.38	0.91 ± 0.08
misonidazole (**5**)	69.2 ± 1.12	2.87 ± 0.67

^{32}P-**9**) were irradiated under anaerobic conditions (freeze−pump−thaw degassed) in the presence of a fixed concentration of *N*-oxide and varying concentrations of thiol. The bimolecular rate constants for the reaction of each *N*-oxide with the C1′-radical were extracted from the slope of the line obtained by plotting yields of labile lesions versus thiol concentration (for example, see Figure 1). Rate constants for the reaction of various *N*-oxides with the C1′-radical in single- and double-stranded DNA oligonucleotides are shown in Table 1.

The rate constant for the reaction of tirapazamine with the C1′-radical in double-stranded DNA (4.6×10^6 M^{-1} s^{-1}) is approximately 50 times lower than that for the same reaction in a single-stranded oligonucleotide (2.5×10^8 M^{-1} s^{-1}). This decreased trapping rate in double-stranded DNA may simply reflect decreased access of the drug to the radical in duplex DNA, where the C1′-position is deeply embedded in the minor groove (*35, 36*). It is interesting to note that reaction of the relatively small molecule β-mercaptoethanol with the C1′-radical in double-stranded DNA is only slightly (∼2-fold) slower than the analogous reaction in single-stranded DNA (*21*).

The mono-*N*-oxide (**3**), which is the major product stemming from the bioreductive metabolism of tirapazamine (*37, 38*), reacts readily with the C1′-nucleotide radical. The rate constant for the reaction of **3** with the radical in single-stranded DNA (4.6×10^8 M^{-1} s^{-1}) is actually slightly higher than that for the parent drug (2.5×10^8 M^{-1} s^{-1}). Placing the radical into duplex DNA slows its reaction with **3** by ∼30-fold, whereas reaction of the parent drug is decreased by a factor of ∼50 in double-stranded DNA. The mono-*N*-oxide (**3**) presents a smaller steric profile than does the parent di-*N*-oxide and, therefore, may enjoy greater access to the minor groove of duplex DNA. In addition, **3** is significantly more hydrophobic than **1** and, thus, may experience greater attraction to the minor groove of duplex DNA. We find that the other possible mono-*N*-oxide derived from the drug, 1,2,4-benzotriazine 4-oxide (**10**), a metabolite formed in the reaction of tirapazamine with the C1′-radical (vide infra), traps the radical in single-stranded DNA ∼3 times less efficiently than **3**, but traps the radical ∼2 times more efficiently than **3** in double-stranded DNA (Table 1).

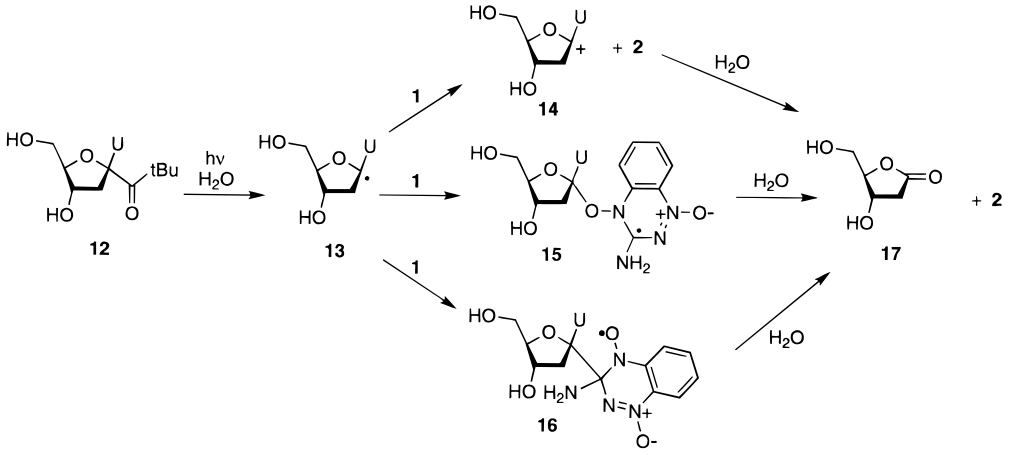
Several substituted quinoxaline 1,4-di-*N*-oxides have been shown in cell culture assays to possess promising hypoxia-selective cytotoxicities similar to tirapazamine (*39*). We have recently found that the parent quinoxaline 1,4-di-*N*-oxide (**11**) can cause radical-mediated, reductively activated DNA cleavage under hypoxic conditions (*40*). Examination of **11**'s ability to substitute for O_2 in radical-mediated DNA damage revealed that the molecule traps the C1′-nucleotide radical in single-stranded DNA with a rate constant of 2.1×10^7 M^{-1} s^{-1}. However, **11** is unable to compete with β-mercaptoethanol (≥1 mM) for the C1′-radical in double-stranded DNA. The simplest heterocyclic *N*-oxide, pyridine *N*-oxide (1 mM), is unable to compete with thiol (≥2 mM) to generate detectable levels of a labile lesion in our experiments.

Deoxygenation of Tirapazamine and Its Metabolites by the C1′-Radical. To better understand the chemistry underlying the *N*-oxide-mediated conversion of the C1′-radical into a base-labile lesion in oligonucleotides, we followed the fate of the tirapazamine and the mono-*N*-oxides **3** and **10** in their reactions with the C1′-nucleoside radical (**13**, Scheme 3). In these reactions, the *N*-oxide and the radical precursor (**12**) were photolyzed in aqueous buffer under anaerobic conditions and the resulting products analyzed by HPLC. The *N*-oxide-derived products were identified by comparison to authentic, synthetically prepared standards.

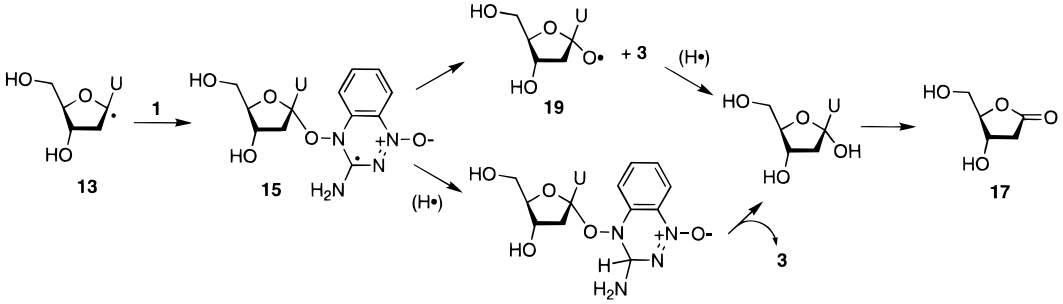
Reaction of tirapazamine with the C1′-radical results in formation of a mixture of both mono-*N*-oxide metabolites (**3** and **10**). While 3-amino-1,2,4-benzotriazine 1-oxide (**3**) is already known as the major metabolite resulting from bioreductive processing of tirapazamine (*37, 38*), the 4-oxide (**10**) has not previously been identified as a tirapazamine metabolite. It is possible that this compound, whose retention time in reverse-phase HPLC chromatography is between that of the drug and the 1-oxide (**3**), corresponds to an uncharacterized drug metabolite of similar chromatographic properties that has been observed in several previous studies (*14, 15*). Reactions of the mono-*N*-oxides **3** and **10** with the C1′-nucleoside radical yield the fully deoxygenated 1,2,4-benzotriazine product (**18**). It is clear from our studies that the reaction of these compounds with the C1′-radical leads to deoxygenation of the *N*-oxide functional group.

The relative importance of C1′-radical oxidation by tirapazamine versus that by its metabolites (**3** and **10**) remains uncertain and will depend on the relative concentrations of each *N*-oxide in the vicinity of DNA. It also remains to be determined whether a *single* drug molecule can efficiently carry out both DNA radical formation and subsequent oxidation of the radical. The expected product of such a

Scheme 3



Scheme 4



reaction, 3-amino-1,2,4-benzotriazine (**18**), is produced during enzymatic metabolism of tirapazamine (*14*), but can arise by various pathways. Participation of a single drug molecule in both reactions might be facilitated by noncovalent association of the drug (or its metabolites) with DNA; however, there is no evidence thus far for such reversible DNA binding by these compounds.

Source of Oxygen in the 2′-Deoxyribonolactone. Conversion of the C1′-radical into a base-labile lesion typically involves oxidation of the radical to the 2′-deoxyribonolactone lesion (**17**, Schemes 3 and 4) (*21*−*23*, *25*, *41*−*43*). Two general mechanisms by which the *N*-oxides studied here might mediate this transformation are depicted for the case of tirapazamine in Schemes 3 and 4. First, the reaction could occur via electron transfer to yield the one-electron-reduced drug (**2**) and the nucleoside cation (**14**), which, following reaction with water, would afford the 2′-deoxyribonolactone (**17**) (Scheme 3). Processes involving formation of unstable covalent drug−C1′ intermediates (e.g., **15** and **16**, Scheme 3), followed by hydrolytic displacement of the drug radical anion from the deoxyribose sugar (hydrolysis at the C1′-carbon), are functionally equivalent to the electron-transfer mechanism. Alternatively, the N−O bond of the oxygen-linked intermediate **15** could homolytically fragment, resulting in direct transfer of the *N*-oxide oxygen to the C1′-radical and formation of the 2′-deoxyribonolactone via the alkoxyl radical **19** (Scheme 4). This process is mechanistically analogous to the production of hydroxyl radical previously proposed (*13*) for the activated form of the drug (**2**, Scheme 1), where the production of an unstable oxygen radical species is thermodynamically driven by rearomatization of the benzotriazine system. The direct transfer of oxygen from the drug to the radical could also proceed via reduction of the intermediate (**15**) followed by elimination (Scheme 4, lower branch).

No covalent adducts of **1** and the monomeric C1′-radical were detected via ^{1}H NMR or electrospray mass spectroscopy. Consequently, isotopic labeling experiments were employed to distinguish between some of the mechanisms described above. By conducting the reactions in $H_2{}^{18}O$, it was possible to determine whether the oxygen found in the 2′-deoxyribonolactone product (**17**) was derived from water (provides ^{18}O 2′-deoxyribonolactone) or directly from the *N*-oxide functional group (yields ^{16}O 2′-deoxyribonolactone). Alternatively, decomposition of any covalent drug−radical adduct during preparation of the GC/MS sample would yield ^{16}O 2′-deoxyribonolactone product. Typically these isotopic tracer experiments were performed by irradiating the monomeric radical precursor **12** with the appropriate *N*-oxide under anaerobic conditions in the absence of thiol, followed by measurement of the ^{16}O:^{18}O content in the bis-TMS ether derivative of **17** using GC/MS.

The reaction of tirapazamine with the C1′-radical occurs predominantly (∼70%) by direct transfer of the *N*-oxide oxygen (^{16}O) to the radical (Table 2), presumably via an unstable covalent intermediate such as **15** (Scheme 4). This result does not support the earlier hypothesis (*44*) that tirapazamine can be efficiently activated (reduced to **2**, as shown in Scheme 3) by deoxyribose radicals, thus resulting in a damage-amplifying radical chain process. Such a damage-amplifying mechanism would produce ^{18}O-containing 2′-deoxyribonolactone. Our experiments should serve as a reasonable test of the notion that deoxyribose radicals can reductively activate **1** because the C1′-radical is likely to be one of the most strongly reducing deoxyribose radicals that can be formed in DNA. The small amount of ^{18}O-lactone

Table 2: Extent of ^{18}O Incorporation in 2′-Deoxyribonolactone (**17**) upon Anaerobic Photolysis of **12** in $H_2{}^{18}O$ in the Presence of Various Drugs

compound	% ^{16}O-**17**
tirapazamine (**1**)	70.2 ± 0.9
3-amino-1,2,4-benzotriazine 1-oxide (**3**)	83.3 ± 0.9
3-amino-1,2,4-benzotriazine 4-oxide (**10**)	18.8 ± 0.5
quinoxaline 1,4-oxide (**11**)	81.0 ± 0.5
TEMPO (**4**)	92.8 ± 3.3
misonidazole (**5**)	41.5 ± 1.2

formed in the reaction of **1** with the C1′-radical may be indicative of a minor pathway in which the DNA radical activates tirapazamine (as shown in Scheme 3). We suspect that the ^{18}O-lactone stems from hydrolysis of intermediates such as **15** or **16** involving attack of water on the C1′ carbon (lower pathways, Scheme 3). In support of this notion, the radical anion of tirapazamine is estimated to have a pK_a ∼ 6 (*44*), suggesting that this species is a reasonably good leaving group.

The percentage of ^{16}O:^{18}O content in **17** produced by the reaction of various *N*-oxides with the C1′-radical (**13**) was also measured (Table 2). The 1-oxide (**3**) and quinoxaline 1,4-di-*N*-oxide (**11**) yield primarily ^{16}O-containing lactone, indicating that these compounds transfer an *N*-oxide oxygen atom directly to the radical via covalent intermediates analogous to **15** (Scheme 4), or via other adducts that decompose during the persilylation reaction. It should be noted that the percent ^{18}O incorporation in the 2′-deoxyribonolactone did not change upon heating the photolysate at 55 °C for 30 min, suggesting that the isotopic partitioning in formation of this product was not a result of a metastable adduct.

Interestingly, the lactone formed by reaction of the DNA radical with the 4-oxide (**10**) predominantly contains ^{18}O from the labeled water. While this finding suggests that the lactone generated by **10** is formed by a mechanism such as those shown in Scheme 3, the reason for the apparent shift in mechanism observed for **10** relative to the other *N*-oxides studied is unclear. It remains possible that reaction of **10** with **13**, like the other *N*-oxides studied, initially generates predominantly ^{16}O-ribonolactone (via direct oxygen transfer, Scheme 4) and that residual 4-oxide in the mixture then serves as a catalyst to accelerate the normally slow (*45*) exchange of ^{18}O from the water into the lactone carbonyl. Catalysis by structurally related *N*-oxides has been suggested in other reactions (*46, 47*).

Reactions of TEMPO and Misonidazole with the C1′-Radical. Our results demonstrate that tirapazamine and its metabolites can serve as surrogates for O_2 in radical-mediated DNA damage reactions. Therefore, we felt it would be useful to compare the reaction of tirapazamine with the C1′-radical to that of representatives from two classes of known "oxygen mimetic" agents (*48, 49*): the nitroxyl radical 2,2,6,6-tetramethylpiperidin-*N*-oxyl (**4**, TEMPO) (*50*) and the nitroimidazole misonidazole (**5**) (*51*). These compounds are known to potentiate the toxic effects of radiation on hypoxic cells due to their ability to substitute for O_2 in radical-mediated damage to biomolecules (*48, 50, 51*). Of special relevance to our studies, misonidazole (*52*) and other nitroaromatics (*53*−*55*) have been reported to increase the yields of single-strand breaks generated upon treatment of cells with ionizing radiation under hypoxic conditions, and, in the case of misonidazole, these strand breaks appear to account for cytotoxic effects of these molecules (*52*).

Misonidazole traps the C1′-nucleotide radical in single- and double-stranded DNA with rate constants of 6.9×10^8 and 2.9×10^7 M^{-1} s^{-1}, respectively. These rates are comparable to those measured for the mono-*N*-oxide metabolites of tirapazamine (**3** and **10**). The 2′-deoxyribonolactone (**17**) derived from reaction of the DNA radical with misonidazole contains approximately equal amounts of ^{16}O and ^{18}O, suggesting that a mixed mechanism is involved in oxidation of the C1′-radical in this experiment. A mechanism for direct transfer of oxygen from the nitro group of misonidazole to a C5′-radical generated by neocarzinostatin has previously been proposed (*56*), and the ^{16}O-lactone in our studies may be derived from an analogous process. The ^{18}O-lactone may be formed via a mechanism analogous to those shown in Scheme 3 except involving reaction at the N−O bonds of misonidazole's nitro group. Similar mechanisms were previously considered for the oxidation of pyrimidin-6-yl radicals by nitrobenzenes (*57*).

The nitroxyl radical-containing compound TEMPO traps the C1′-radical in single- and double-stranded DNA with rate constants of 7.2×10^7 and 9.1×10^6 M^{-1} s^{-1}, respectively. While these rates are markedly lower than the known rates for reaction between TEMPO and simple alkyl radicals in organic solvents (typically $\sim 1 \times 10^9$ M^{-1} s^{-1}), they are comparable to the lower rates observed for the reaction of nitroxides with resonance-stabilized radicals in polar, hydroxylic media (*58*).

The 2′-deoxyribonolactone derived from reaction of the C1′-nucleoside radical with TEMPO contains almost exclusively ^{16}O. This suggests that the expected C1′-nitroxide adduct forms and cleaves in situ or during the persilylation reaction. Although it has been reported that nitroxyl radical adducts can be thermally unstable (*59*), heating the photolysate at 55 °C for 30 min did not alter the level of ^{18}O incorporation in the product.

Nitroxyl radicals have been described both as hypoxic radiosensitizers and as radioprotective agents. The radioprotective properties of nitroxyl radicals may stem from their ability to directly scavenge DNA-damaging radicals or to interfere with metal-mediated generation of reactive oxygen species (*60, 61*). On the other hand, the radiosensitizing properties of nitroxyl radicals are thought to stem from their ability to substitute for O_2 in reactions with radiolytically generated radicals (*62*, *63*). It has been suggested that covalent nitroxyl radical−DNA adducts may be responsible for the observed hypoxic radiosensitization of mammalian cells (*63*), though it has been noted that these agents cause only a small increase in the yield of radiation-induced, spontaneous single-strand breaks under low-oxygen conditions. The chemistry reported here, indicating that the nitroxyl radical TEMPO may convert the C1′-radical to a base-labile lesion via a direct oxygen-transfer reaction involving a covalent intermediate, may provide a chemical basis for understanding previously observed radiobiological effects of nitroxyl radicals.

CONCLUSIONS

The chemical properties of the hypoxia-selective antitumor agent tirapazamine (**1**) that may allow this agent to efficiently

cleave DNA under low-oxygen conditions have been characterized. Tirapazamine and its mono-*N*-oxide metabolites (**3**, **10**) can serve as surrogates for molecular oxygen in radical-mediated cleavage of single-stranded and double-stranded DNA. These results, along with previous reports (*13*), demonstrate that the drug can initiate the formation of deoxyribose radicals in DNA *and* convert those radicals into toxic strand damage events under low-oxygen conditions. Such reactions of the drug and its metabolites with DNA radicals may increase the efficiency of drug-mediated DNA damage and potentially play an important role in determining the chemical structure of the strand damage products. In addition, our data indicate that reactions of tirapazamine and its metabolites with radical lesions in DNA should be reconsidered (*64*) as a possible contributor to the clinically observed synergistic action of tirapazamine with radiation therapy (*6, 9, 64*).

It is important to critically evaluate the possible in vivo relevance of the reaction between tirapazamine and DNA radicals. A primary consideration in this regard is the relative rates at which DNA radicals are trapped by thiol versus the drug. The relative efficiencies of these two pathways are dictated by the respective second-order rate constants and by the in vivo concentrations of thiol and drug. The rate constants for the reaction of tirapazamine, its mono-*N*-oxide metabolite (**3**), and the thiol 2-mercaptoethanol with a C1′-radical in duplex DNA are 4.5×10^6, 1.4×10^7, and 1.8×10^6 M^{-1} s^{-1}, respectively. These roughly comparable rate constants, combined with the relatively high concentrations of the endogenous thiol glutathione present in the cell (*19, 65*), initially might lead one to suspect that tirapazamine and its metabolites would not be capable of successfully competing with thiol for reaction at DNA radicals.

However, further consideration suggests that the reaction between these *N*-oxides and DNA radicals could be significant. First, the biological thiol glutathione traps DNA radicals less efficiently than the simple, uncharged thiol 2-mercaptoethanol used in our studies (*20*). Second, the C1′-radical examined in our current studies is relatively inaccessible in duplex DNA (*35, 36*). The 50-fold decrease in the rate constant for the reaction of tirapazamine with the C1′-radical in duplex DNA versus single-stranded DNA is consistent with this point. Reaction of tirapazamine and its metabolites at the more accessible C4′- and C5′-positions in duplex DNA may be more efficient than the reaction with the C1′-radical that we have studied here. Thus, the rate constants for reaction of tirapazamine and its metabolites at the C4′- and C5′-positions in duplex DNA might be comparable to those observed for reaction at the C1′-position in single-stranded DNA [$(2-5) \times 10^8$ M^{-1} s^{-1}]. Further investigations will be required to examine this possibility. Third, the superhelical state and association with nuclear proteins can alter the access of thiols and drugs to DNA in vivo (*66*−*68*). For example, positive supercoiling is known to limit the accessibility of glutathione to the double helix while not affecting binding by calicheamicin (*69*). This supports the notion that, access of tirapazamine to some regions of DNA in vivo could be favored over that for glutathione, thus facilitating trapping of DNA radicals by the drug rather than thiol. Finally, although somewhat lower than the rate constant reported for trapping of the C1′-radical by O_2 (1×10^9 M^{-1} s^{-1}) (*70*), it is noteworthy that the rate constants for the trapping of this DNA radical by tirapazamine and its metabolites are comparable to those measured for misonidazole, a compound that is known to derive biological activity (at least in part) through its ability to substitute for O_2 in radical-mediated strand scission reactions (*51*).

Further studies of this unprecedented reaction involving transfer of oxygen from heterocyclic *N*-oxides to a carbon-centered radical are underway to determine the detailed mechanism and the structural features required in the *N*-oxide substrate.

SUPPORTING INFORMATION AVAILABLE

Detection of deoxygenated products resulting from the reaction of *N*-oxides (**1**, **3**, and **10**) with **13** (13 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI991488N