Direct Evidence for Bimodal DNA Damage Induced by Tirapazamine

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The ability of tirapazamine (**1**, 3-amino-1,2,4-benzotriazine 1,4-dioxide, SR4233) to fix DNA radical lesions is demonstrated by studying the reaction between the antitumor drug and an oligonucleotide radical that is independently produced at a defined site within a biopolymer. Using β-mercaptoethanol as a competitor, it was determined that tirapazamine traps a C1′-nucleotide radical with a rate constant of $\sim 2 \times 10^8$ M^{-1} s^{-1}. Product and isotopic labeling studies suggest that tirapazamine reacts with the radical via covalent adduct formation, resulting primarily from reaction at the *N*-oxide oxygen. Intermediate covalent adducts could not be observed, but are postulated to decompose to the alkaline labile 2′-deoxyribonolactone lesion. These experiments affirm recent proposals suggesting that tirapazamine can serve as a surrogate for O_2 in converting DNA radicals into toxic strand damage events.

Tirapazamine (**1**, 3-amino-1,2,4-benzotriazine 1,4-dioxide, SR4233) is the lead member of a family of heterocyclic *N*-oxides that exhibit promising antitumor properties (*1*−*4*) and is currently undergoing clinical trials for the treatment of various cancers (*3*). The benzotriazine 1,4-dioxides are exciting potential therapeutic agents, in part because they show selective cytotoxicity against the oxygen-poor (hypoxic) cells found in tumors (*1*−*4*). The selectivity of tirapazamine in a variety of cell lines is superior to that exhibited by many hypoxia-selective cytotoxins such as mitomycin C and misonidazole (*3*, *4*). DNA damage caused by tirapazamine accounts for its cytotoxicity (*3*, *5*). The drug undergoes bioreductive activation, and the one electron-reduced form (**2**) damages DNA via a free radical mechanism (*3*, *4*). It has been proposed that DNA cleavage by tirapazamine may be attributable, at least in part, to release of hydroxyl radical (Scheme 1) (*6*). The selectivity for hypoxic cells is derived from the rapid deactivation of the one-electron-reduced drug by O_2.

Evidence was recently presented supporting a hypothesis in which tirapazamine plays two roles in inducing DNA damage under hypoxic conditions (*7*). It was proposed that, in addition to initiating DNA strand damage, **1** substitutes for O_2 by trapping the C4′-radical intermediate. This proposal stems from the observation that the amount of DNA strand cleavage products containing 3′-phosphoglycolate termini formed in samples subjected to γ-radiolysis under anaerobic conditions was increased by the presence of tirapazamine. 3′-Phosphoglycolate termini are generally considered a signature product for C4′-hydrogen atom abstraction under aerobic conditions (*8*, *9*); thus, it was suggested that the drug donates an oxygen atom to the deoxyribosyl radical (*7*). We hypothesized that **1** may trap DNA radicals in general. Thus, to better understand the chemical processes underlying the biological action of this promising drug, we investigated the interaction of tirapazamine with the C1′-DNA radical, a key intermediate in DNA damage induced by a variety of agents, including hydroxyl radical (*10*).

We report herein results of experiments that clearly support the ability of tirapazamine to serve as a surrogate for O_2 by trapping C1′-radicals in DNA. Using the ability to independently generate a reducing deoxyribosyl radical at a defined site in an oligonucleotide (*10*), we show that the drug (**1**) competes with β-mercaptoethanol for a C1′-radical, producing alkaline labile lesions in the absence of O_2. The cytotoxicity of radical-based DNA-damaging agents is typically decreased under low-oxygen conditions due to the fact that many DNA radical lesions require reaction with O_2 to convert the radical into toxic DNA modifications. Our results provide direct evidence for the trapping of a DNA radical by tirapazamine, and offer insight regarding the chemical mechanisms by which this antitumor agent can efficiently damage DNA via radical intermediates under hypoxic conditions. The interaction of tirapazamine with DNA radicals that we report here may also play a role in the synergistic action of this drug with fractionated radiation that has been observed in the clinic (*3*, *11*).

Experimental Procedures

Materials. Compounds **1**, **5**, and **10** were synthesized as previously described (*10*, *12*, *13*). ^{32}P-labeling was carried out with T4 polynucleotide kinase following standard procedures (*14*). Oligonucleotides were sequenced using a reaction specific for 2′-deoxyadenosine (*15*). [γ-^{32}P]ATP was from Amersham. Phosphorimaging was carried out on a Molecular Dynamics phosphorimager equipped with ImageQuant software.

Anaerobic Photolysis Reactions. Pyrex photolysis tubes were loaded with the appropriate reagents (e.g., **1**, **5**, **10**, and β-mercaptoethanol); the samples were degassed with three freeze−pump−thaw cycles (3 min each) and the tubes sealed

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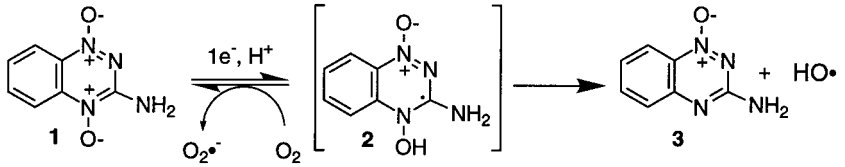
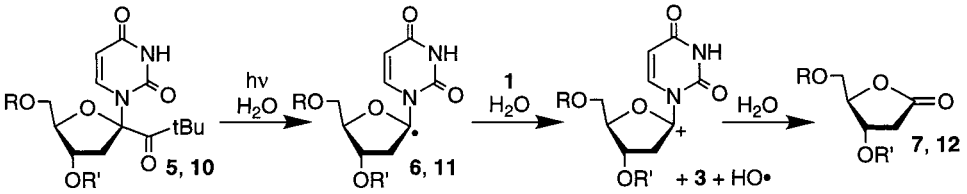
Scheme 1

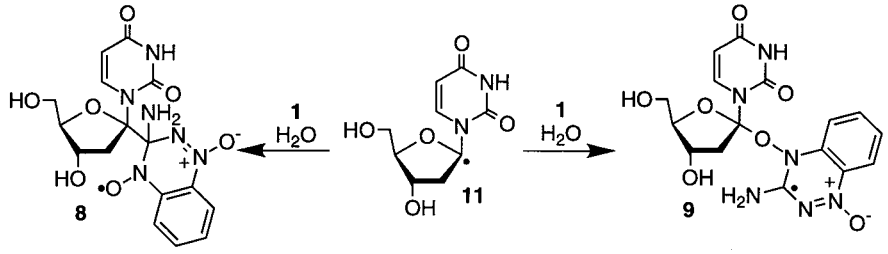


Scheme 2



5, 6, 7 R = 5'-d(GTC ACG TGC TGC Ap), R' = 5'-d(pACG ACG TGC TGA GCC T)
10, 11, 12 R = R' = H

Scheme 3

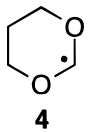


under vacuum. Photolyses were carried out for 20 min in Pyrex tubes (6 mm i.d.) using a Rayonet photoreactor equipped with 16 lamps (λ_{max} = 350 nm).

Alkaline Labile Lesion Determination. [5′-^{32}P]**5** in phosphate buffer (pH 7.0, 10 mM), NaCl (100 mM), **1** (1 mM), and the appropriate amount of β-mercaptoethanol (1−50 mM) were loaded in Pyrex tubes as described above, and the mixture was photolyzed. After photolysis, the samples were transferred to Eppendorf vials, the photolysis tubes were washed with H_2O (25 μL), and the DNA was precipitated using standard protocols (*14*). The DNA was resuspended in 10% (by volume) aqueous piperidine (100 μL) and held at 95 °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. The samples were analyzed using 20% denaturing polyacrylamide gel electrophoresis. Gels were run until the BPB dye was 1 in. from the bottom of the plate. Quantification of the lesions was accomplished by exposing the gels to a Molecular Dynamics storage phosphor screen for 15 h, followed by analysis on a Molecular Dynamics phosphorimager. Qualitative analysis was carried out using X-ray films, with a typical exposure time of 12 h at −80 °C with an intensifier screen.

Results and Discussion

At the outset of these experiments, two general mechanisms by which **1** might fix DNA lesions were considered. Although the electrochemistry of the C1′-nucleotide radical has not been reported, model studies on **4** suggested that the oxidation potential of this molecule could be as low as 0.3 V (*16*).



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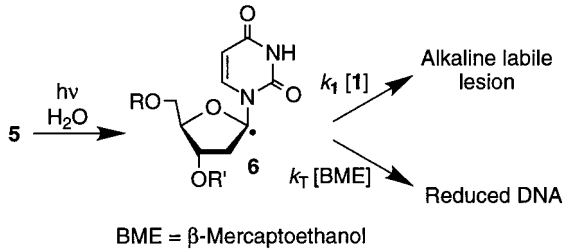
If so, generation of **6** under anaerobic conditions in the presence of tirapazamine (E_{red} = −0.45 V) (*17*) could result in electron transfer from the radical to the drug (Scheme 2), provided this unfavorable process is followed by an irreversible reaction (*18*). The alkaline labile lesion (**7**) produced by such a reaction would be the same as that formed in the presence of O_2 (*19*). In addition, this process could result in formation of the one-electron-reduced form of the drug in the vicinity of the oligonucleotide, causing DNA damage amplification (*20*, *21*) due to the release of hydroxyl radical (*6*). Alternatively, trapping of the C1′-radical by **1** on either carbon (**8**) or oxygen (**9**) would result in fixing the lesion (Scheme 3). The pathways shown in Schemes 2 and 3 require bimolecular interaction between the DNA radical and tirapazamine, and result in the formation of an alkaline labile lesion (**7**) at the site of radical generation. Consequently, it was anticipated that fixing of a DNA radical lesion by tirapazamine could be detected by determining whether the drug can compete with β-mercaptoethanol for reaction with the C1′-radical **6**. It was previously shown that low concentrations of β-mercaptoethanol (0.5 mM) efficiently trap the C1′-nucleotide radical under anaerobic conditions (*10*), forming a mixture of α- and β-deoxyuridine (*22*). The rate constant for trapping the C1′-nucleotide radical in DNA by β-mercaptoethanol is ∼1.1 × 10^7 M^{-1} s^{-1} (*10*). Hence, determination of the amounts of alkaline labile lesions as a function of thiol concentration in the presence of a constant amount of tirapazamine can yield the overall rate constant for reaction of the drug with the C1′-nucleotide radical (Scheme 4, eq 1).

$$\frac{\text{cleavage of } \mathbf{1}}{\text{trapping of BME}} = \frac{k_1[\mathbf{1}]}{k_T}\left(\frac{1}{[\text{BME}]}\right) \qquad (1)$$

Scheme 4



BME = β-Mercaptoethanol

Irradiation of freeze−pump−thaw degassed samples of [5′-^{32}P]**5** in the presence of 1 mM tirapazamine produced yields of alkaline labile lesions that depended inversely on the concentration of β-mercaptoethanol (Figure 1). Using the previously determined (*10*) rate constant for trapping of **6** by β-mercaptoethanol, a bimolecular rate constant of $\sim 2.0 \times 10^8$ M^{-1} s^{-1} for the reaction of the drug with the C1′-DNA radical was extracted from this linear plot (eq 1). The rate constant is comparable to those measured for the reactions of nitroxides (e.g., Tempo) with alkyl radicals (*23*). Although this bimolecular rate constant is approximately 1 order of magnitude smaller than that for trapping by O_2, it indicates that sub-millimolar concentrations of tirapazamine can effectively compete with physiological levels of thiol for a DNA radical.

Investigations into the mode of reaction between **6** and tirapazamine were carried out using monomer and polymer probes. The viability of the electron-transfer pathway was examined by searching for signs of HO• production (Schemes 1 and 2). Spin trapping was not attempted, because this method had proven ineffective in previous studies on 2′-deoxyuridin-1′-yl (*19*). Examination of the oligonucleotide cleavage pattern (data not shown) does not indicate a higher degree of background cleavage at low (or no) thiol concentrations which would be attributable to formation of freely diffusible HO•. In addition, anaerobic photolysis of **10** in the presence of tirapazamine and plasmid DNA did not give rise to a significant amount of relaxed circularized plasmid above background. These results suggest that significant amounts of hydroxyl radical are not produced as a result of the reaction of the drug with **6**.

Using monomer **10** as a probe, product studies and isotopic labeling were used to further address the mechanism of reaction between 2′-deoxyuridin-1′-yl (**11**) and the drug **1** (Scheme 5). When **10** was irradiated under anaerobic conditions in the presence of tirapazamine, **12** was detected via GC/MS as its bis-TMS ether derivative (*19*, *24*). No covalent adducts of **1** and **11** were detected via this analytical method, or by analysis of the crude photolysate by electrospray mass spectrometry. However, production of **3** mediated by **11** was observed by GC/MS and HPLC when **1** was irradiated in the presence

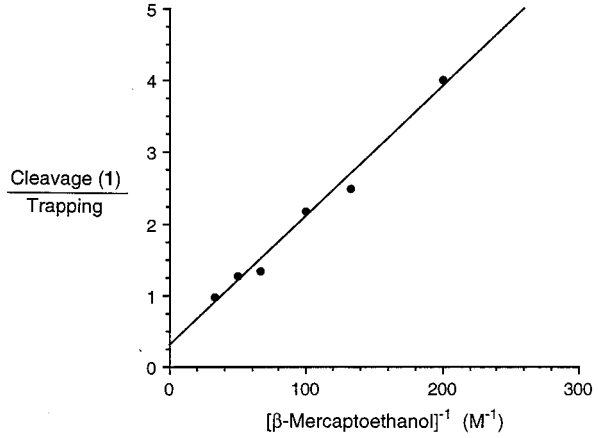
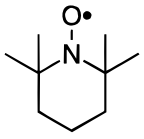


Figure 1. Determination of the effect of β-mercaptoethanol (BME) on the formation of alkaline labile lesions from 2′-deoxyuridin-1′-yl (**6**) in the presence of tirapazamine (1 mM).

of **10**. To distinguish between electron transfer and trapping of the radical by tirapazamine, photolyses were carried out under anaerobic conditions in $H_2{}^{18}O$. Trapping of the carbocation produced via the electron-transfer pathway would yield [^{18}O]**12** (*19*). In contrast, formation of **12** via covalent adduct formation could give rise to either [^{16}O]**12** or [^{18}O]**12**, depending upon whether trapping occurs on oxygen or carbon, respectively. In the event, irradiation of **10** and the drug in $H_2{}^{18}O$ gave rise to a predominance of [^{16}O]**12**, whose percentage (83 ± 2%) did not vary as a function of thiol concentration (1−50 mM). Observation of [^{16}O]**12** as the major product suggests that oxygenation of **11** occurs by direct transfer of oxygen from tirapazamine to the DNA radical via an intermediate such as **9**, which collapses to **12** in situ (Scheme 3).

To our knowledge, the products resulting from reactions of benzotriazine di-*N*-oxides and alkyl radicals have not been reported; in general, however, the reaction of carbon-centered radicals at the oxygen of *N*-oxides is not a well-known reaction. For example, such radicals commonly react with pyrroline *N*-oxides via carbon−carbon bond formation and undergo addition to the aromatic ring of simple heterocyclic *N*-oxides (*25*). The reactivity of the N−O bond of tirapazamine in the oxygen transfer reaction described here is more reminiscent of nitroxides, such as Tempo (**13**), or nitroso compounds,



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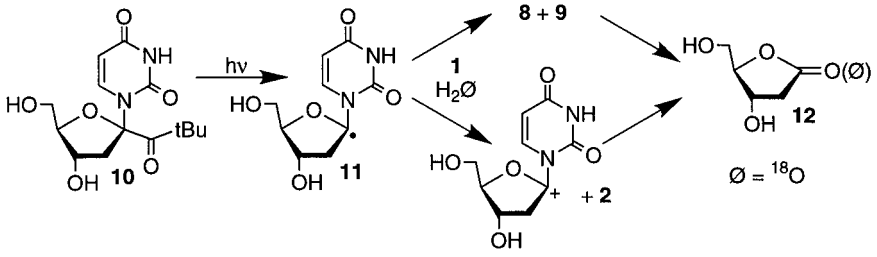
both of which can react with radicals at the oxygen atom of their N−O bonds (*23*, *26*).

Scheme 5



The relatively small amount of ^{18}O-containing **12** formed during the photolysis of **10** could be derived from either the electron-transfer (Scheme 2) or carbon atom trapping pathway (**8**, Scheme 3). On the basis of the negative results above concerning the formation of hydroxyl radical, we believe that the existence of the electron-transfer pathway is unlikely. In the absence of any other evidence, we tentatively suggest that [^{18}O]**12** is formed via trapping of **11** by the tirapazamine on the carbon atom of the drug followed by thiol-mediated reduction and solvolysis.

Conclusions

We have provided the first evidence using an independently generated radical in an oligonucleotide that tirapazamine fixes DNA lesions by reacting with radical lesions produced in the biopolymer. These experiments demonstrate that, like the radiosensitizer misonidazole (*27*), tirapazamine traps DNA radicals to produce alkaline labile lesions. In addition, our studies confirm the dual nature of this antitumor agent in causing DNA damage.

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Supporting Information Available: Plot of the [^{16}O]**12**:[^{18}O]**12** ratio from irradiation of **10** in the presence of **1** as a function of β-mercaptoethanol concentration and experimental procedures and results for the irradiation of **10** in the presence of **1** and plasmid DNA (5 pages). Ordering information is given on any current masthead page.

References

(1) Zeman, E. M., Brown, J. M., Lemmon, M. J., Hirst, V. K., and Lee, W. W. (1986) SR4233: A new bioreductive agent with high selective toxicity for hypoxic mammalian cells. *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 1239−1242.

(2) Zeman, E. M., Baker, M. A., Lemmon, M. J., Pearson, C. I., Adams, J. A., Brown, J. M., Lee, W. W., and Tracy, M. (1989) Structure−activity relationships for benzotriazine di-*N*-oxides. *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 977−981.

(3) Brown, J. M., and Siim, B. G. (1996) Hypoxia-selective cytotoxins in cancer therapy. *Semin. Radiat. Oncol.* **6**, 22−36.

(4) Brown, J. M. (1993) SR4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. *Br. J. Cancer* **67**, 1163−1170.

(5) Siim, B. G., van Zijl, P. L., and Brown, J. M. (1996) Tirapazamine-induced DNA damage measured using the comet assay correlates with cytotoxicity towards hypoxic tumour cells in vitro. *Br. J. Cancer* **73**, 952−960.

(6) Daniels, J. S., and Gates, K. S. (1996) DNA cleavage by the antitumor agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (SR4233): evidence for involvement of hydroxyl radical. *J. Am. Chem. Soc.* **118**, 3380−3385.

(7) Jones, G. D. D., and Weinfeld, M. (1996) Dual action of tirapazamine in the induction of DNA strand breaks. *Cancer Res.* **56**, 1584−1590.

(8) Stubbe, J., and Kozarich, J. W. (1987) Mechanisms of bleomycin-induced DNA degradation. *Chem. Rev.* **87**, 1107−1136.

(9) Giese, B., Beyrich-Graf, X., Erdmann, P., Giraud, L., Imwinkelried, P., Müller, S. N., and Schwitter, U. (1995) Cleavage of single-stranded 4′-oligonucleotide radicals in the presence of O_2. *J. Am. Chem. Soc.* **117**, 6146−6147.

(10) Tronche, C., Goodman, B. K., and Greenberg, M. M. (1998) DNA damage induced via independent generation of the radical resulting from formal hydrogen atom abstraction from the C1′-position of a nucleotide. *Chem. Biol.* **5**, 263−271.

(11) Brown, J. M., and Lemmon, M. J. (1990) Potentiation by the hypoxic cytotoxin SR4233 of cell killing produced by fractionated irradiation of mouse tumors. *Cancer Res.* **50**, 7745−7749.

(12) Mason, J. C., and Tennant, G. (1970) Heterocyclic *N*-oxides. Part IV. Synthesis and nuclear magnetic resonance spectra of 3-aminobenzo-1,2,4-triazines and their mono- and di-*N*-oxides. *J. Chem. Soc. B*, 911−916.

(13) Greenberg, M. M., Yoo, D. J., and Goodman, B. K. (1997) C1′ Acylated derivatives of 2′-deoxyuridine. Photolabile precursors of 2′-deoxyuridin-1′-yl. *Nucleosides Nucleotides* **16**, 33−40.

(14) Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular cloning a laboratory manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.

(15) Iverson, B. L., and Dervan, P. B. (1987) Adenine specific DNA chemical sequencing reaction. *Nucleic Acids Res.* **15**, 7823−7830.

(16) Wayner, D. D. M., and Griller, D. (1989) Electrochemistry of transient free radicals. *Mol. Struct. Energ.* **11**, 109−128.

(17) Priyadarsini, K., Indira, K., Tracy, M., and Wardman, P. (1996) The one-electron reduction potential of 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine): A hypoxia-selective bioreductive drug. *Free Radical Res.* **25**, 393−399.

(18) Eberson, L. (1987) *Electron-transfer reactions in organic chemistry*, Springer-Verlag, Berlin.

(19) Tallman, K. A., Tronche, C., Yoo, D. J., and Greenberg, M. M. (1998) Release of superoxide from nucleoside peroxyl radicals, a double-edged sword? *J. Am. Chem. Soc.* **120**, 4903−4909.

(20) Frankenberg-Schwager, M., and Frankenberg, D. (1990) DNA double-strand breaks: their repair and relationship to cell killing in yeast. *Int. J. Radiat. Biol.* **58**, 569−575.

(21) Greenberg, M. M., Barvian, M. R., Cook, G. P., Goodman, B. K., Matray, T. J., Tronche, C., and Venkatesan, H. (1997) DNA damage induced via 5,6-dihyrothymid-5-yl in single-stranded oligonucleotides. *J. Am. Chem. Soc.* **119**, 1828−1839.

(22) Goodman, B. K., and Greenberg, M. M. (1996) Independent generation and reactivity of 2′-deoxyurid-1′-yl. *J. Org. Chem.* **61**, 2−3.

(23) Bowry, V. W., and Ingold, K. U. (1992) Kinetics of nitroxide radical trapping. 2. structural effects. *J. Am. Chem. Soc.* **114**, 4992−4996.

(24) Dizdaroglu, M. (1990) Gas chromatography−mass spectrometry of free radical-induced products of pyrimidines and purines in DNA. *Methods Enzymol.* **193**, 842−857.

(25) Elofson, R. M., Gadallah, F. F., and Schulz, K. F. (1971) Homolytic arylation of pyridine and pyridine *N*-oxide and the effect of localization energy and temperature on arylation patterns. *J. Org. Chem.* **36**, 1526−1531.

(26) Terabe, S., and Konaka, R. (1973) Spin trapping by use of nitroso-compounds. Part V. 2,4,6-tri-*tert*-butyl-nitrosobenzene: a new type of spin-trapping reagent. *J. Chem. Soc., Perkin Trans. 2*, 369−374.

(27) Kappen, L. S., Lee, T. R., Yang, C.-c., and Goldberg, I. H. (1989) Oxygen transfer from the nitro group of a nitroaromatic radiosensitizer to a DNA sugar damage product. *Biochemistry* **28**, 4540−4542.

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