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Oxidative DNA Base Damage by the Antitumor Agent 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (Tirapazamine)

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Abstract—Tirapazamine is a bioreductively activated DNA-damaging agent that selectively kills the hypoxic cells found in solid tumors. In this work, base excision repair enzymes were used to provide evidence that tirapazamine causes significant amounts of damage to both purine and pyrimidine residues in double-stranded DNA.

The heterocyclic *N*-oxide 3-amino-1,2,4-benzotriazine 1,4-dioxide (**1**, tirapazamine) is a bioreductively activated DNA-damaging agent that selectively kills the oxygen-poor (hypoxic) cells found in solid tumors.[1,2] Accordingly, the compound shows promise as an antitumor agent and is currently undergoing various phase I, II, and III clinical trials for the treatment of several human cancers.[2] Tirapazamine is enzymatically reduced in cells to yield a crucial radical intermediate (**2**, Scheme 1).[3] In normally oxygenated cells, the activated form of the drug (**2**) is rapidly destroyed by reaction with O_2,[3–5] but, in the hypoxic environment of tumors, this radical goes on to cause oxidative cleavage of the DNA backbone.[3,6,7] A wealth of experimental data indicates that DNA is an important cellular target for tirapazamine;[8,9] however, the exact nature of the chemical species responsible for tirapazamine-mediated strand cleavage remains uncertain. It is commonly suggested that the tirapazamine radical (**2**) directly abstracts hydrogen atoms from the DNA backbone;[1,2] however, there is evidence indicating that the N-OH bond in the protonated tirapazamine radical (**2**) may undergo homolytic fragmentation to release the well known DNA-cleaving agent hydroxyl radical (as shown in Scheme 1).[7,10] Recent studies show that, in addition to *generating* deoxyribose radicals, tirapazamine (and its mono-*N*-oxide metabolites) can further *react with* these DNA radicals, thus converting them into strand breaks.[11–13]

We were intrigued by a recent report suggesting that activated tirapazamine selectively damages the deoxyribose sugars of DNA but *does not* react with the heterocyclic bases in the biopolymer.[13] We found this surprising because, typically, small diffusible agents that have the oxidizing power to abstract hydrogen atoms from the deoxyribose backbone also cause oxidative damage to the bases in DNA.[14] For example, when hydroxyl radical (HO•) attacks double-stranded DNA, substantial amounts of base damage occur along with reactions at the sugar-phosphate backbone of the biopolymer.[14–19] In light of the expectation that tirapazamine will cause DNA base damage, and with the knowledge that base modification can have serious biological consequences,[20–22] we decided to investigate whether this drug damages the heterocyclic bases in duplex DNA. In the work reported here, we have employed base excision repair enzymes to provide evidence that the antitumor agent tirapazamine does, in fact, cause significant amounts of damage to both purine and pyrimidine residues in double-stranded DNA.

DNA repair endonucleases are useful tools for the analysis of DNA damage.[23,24] Used in this context, repair enzymes do not *repair* damaged DNA at all; rather, they recognize and cleave DNA at particular lesions, leaving behind a strand break that can be readily detected using polyacrylamide or agarose gel electrophoresis. Each repair enzyme used in this study recognizes and cleaves at a *set* of DNA lesions (the substrate specificities are summarized in Table 1).[23] The enzymes exonuclease III (exo III) and endonuclease IV (endo IV) reveal damage to the DNA backbone by converting

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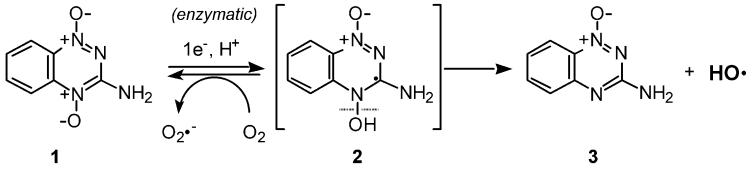


Scheme 1.

Table 1. Substrate specificities for repair enzymes used in this study[a]

Enzyme	AP Sites			Oxidized bases	
	Regular	1′-Ox	4′-Ox	Purines	Pyrimidines
Exo III	+	+	–	–	–
Endo IV	+	+	+	–	–
FPG	+	–	+	+	–
Endo III[b]	+	–	+	–	+

[a]For a more complete version of this table, see ref 23.
[b]Endo III has recently been shown to process some purine lesions.[30]

C1′-oxidized sugar residues (exo III and endo IV), C4′-oxidized sugar residues (endo IV), and abasic sites (exo III and endo IV) into strand breaks (Fig. 1).[23,25,26] The base excision repair enzymes employed in this work, endonuclease III (endo III) and formamidopyrimidine glycosylase (FPG), remove a wide variety of oxidatively damaged pyrimidine and purine bases, respectively, from duplex DNA (Fig. 1) and also catalyze subsequent strand cleavage at the resulting abasic site (Scheme 2).[27–30] The strand breaks caused by the reagents used in this study provide a quantitative indication that a certain type of lesion was present in the treated DNA. When DNA damaged by a particular agent is treated with a panel of repair enzymes, the resulting response (relative number of strand breaks induced by each enzyme) has been referred to as the 'endonuclease fingerprint' for that DNA-damaging agent.[23,24] The endonuclease fingerprint obtained for a given agent provides information regarding the general types of DNA lesions that are formed and, when compared to the endonuclease fingerprint of other well characterized DNA-damaging agents, can provide insight regarding the chemical nature of the species responsible for the DNA damage.[24]

We used a plasmid-based, agarose gel assay to measure the strand breaks induced by endonuclease treatment of tirapazamine-damaged DNA. This assay measures DNA strand breaks by monitoring the relative amounts of uncut (supercoiled, form I) and cleaved (nicked, form II) plasmid DNA present in each reaction. With this assay it is possible to quantitatively detect the increase in number of strand breaks caused by treatment of the damaged DNA with various repair enzymes. The compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (**1**, tirapazamine) was prepared according to the method of Fuchs et al.[31] Tirapazamine was activated using either the NADPH:cytochrome P450 oxidoreductase or the xanthine/xanthine oxidase enzyme system under anaerobic conditions. Both of these systems have been shown to effectively trigger DNA strand cleavage by tirapazamine[3,6,7] and are known to generate the same spectrum of metabolites obtained from in vivo metabolism of the drug.[31] The NADPH:cytochrome P450 oxidoreductase system is thought to be the major enzyme responsible for the bioactivation of tirapazamine.[32] All DNA-damage reactions were prepared using degassed solutions in an inert atmosphere glove bag and incubated under anaerobic conditions protected from light.[33]

Figure 2 shows the endonuclease fingerprint for DNA damage by the tirapazamine-NADPH:cytochrome P450 oxidoreductase system under anaerobic conditions. Tirapazamine causes spontaneous strand breaks in the DNA under these conditions and these direct strand

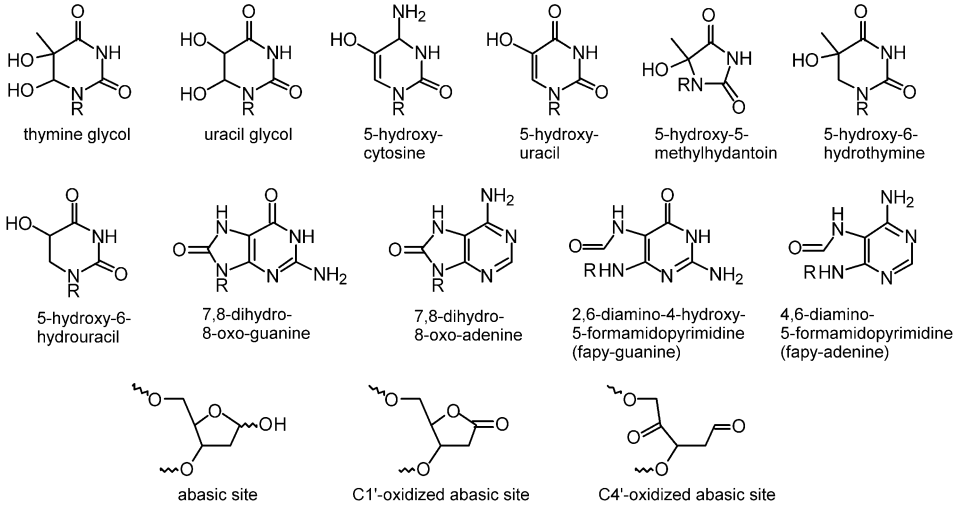
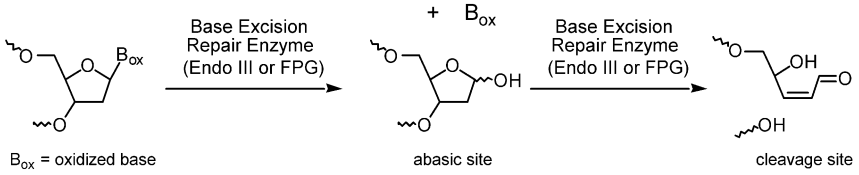


Figure 1. Structures of representative oxidatively-damaged DNA substrates recognized by the enzymes used in this study.



Scheme 2.

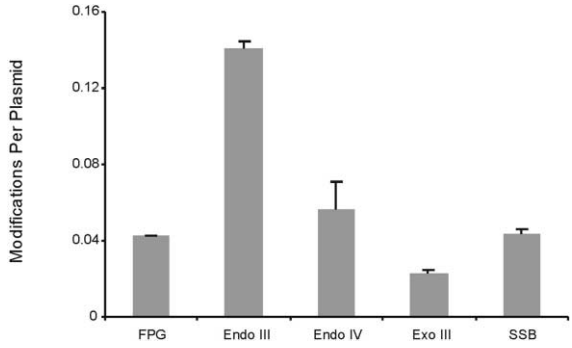


Figure 2. Endonuclease fingerprint for tirapazamine-mediated DNA damage (activated by NADPH:cytochrome P450 oxidoreductase under hypoxic conditions). DNA damage reactions with tirapazamine were conducted as described previously.[36] The solutions contained 1 μg of supercoiled pGL2 DNA, 500 μM NADPH, 50 mM sodium phosphate (pH 7.0), 1 mM desferal, 2 mUnits of cytochrome P450 oxidoreductase, 0.2 mg/mL catalase, and 12.5 μM tirapazamine in 30 μL total volume. Catalase and the iron chelator desferal were included to quench potential background oxidative DNA damage stemming from the conversion of trace amounts of molecular oxygen to superoxide radical by either the enzyme or tirapazamine radical anion. This mixture was incubated for 4 h and the DNA then precipitated by addition of 500 μL of 0.3 M sodium acetate (pH 5.2) in 70% ethanol, the resulting pellet washed with 70% EtOH (500 μL), and the DNA pellets were briefly air dried. The pellets were subsequently redissolved in the appropriate buffer and treated with repair endonucleases as described in ref 37. The tirapazamine reaction conditions were carefully optimized to maintain levels of supercoiled and nicked plasmid within a range that allows reliable quantitation from the agarose gels (no linearized DNA present).

breaks are depicted by the height of the bar labeled 'SSB' on the plot. The bar heights shown for each endonuclease in Figure 2 represent the *increase*, above and beyond spontaneous strand breaks, that results from enzyme treatment. The total number of strand breaks following exo III treatment (enzyme-induced *plus* spontaneous cleavage events) is 1.5±0.1-fold greater than the number of strand breaks present in the tirapazamine-damaged DNA prior to enzyme treatment. The exo III-mediated increase in strand breaks indicates that abasic sites and/or C1′-oxidized abasic sites are formed by tirapazamine. Treatment of the DNA with endo IV affords a somewhat larger increase in the number of strand breaks (2.3±0.1-fold increase), indicating that C4′-oxidized sugar residues are produced in the DNA damage reaction. The increase in strand cleavage catalyzed by treatment with FPG (2.0±0.1-fold increase) suggests that oxidized purine residues are present in tirapazamine-damaged DNA. Treatment of drug-damaged DNA with endo III results in the largest increase in strand breaks (4.2±0.1-fold increase), indicating that damaged pyrimidine residues probably constitute the dominant form of base damage caused by activated tirapazamine.

Activation of tirapazamine by xanthine/xanthine oxidase under hypoxic conditions yields an endonuclease fingerprint (Fig. 3) very similar to that shown for activation by the NADPH:cytochrome P450 oxidoreductase system (Fig. 2). This outcome was anticipated because both enzymes generate the same reactive intermediate (**2**) from tirapazamine. When the drug is activated by xanthine oxidase, treatment of the damaged DNA by exo III yields a 1.4±0.2-fold increase in strand breaks, endo IV yields a 1.8±0.2-fold increase, exposure to FPG yields a 1.7±0.3-fold increase, and endo III a 2.8±0.2-fold increase.

As a point of reference, we felt it would be informative to compare the endonuclease fingerprint of enzymatically-activated tirapazamine (Figs. 2 and 3) with that of authentic hydroxyl radical under hypoxic conditions (Fig. 4). It is well known that hydroxyl radical causes oxidative base damage along with spontaneous strand cleavage under anaerobic conditions;[14–17] however, to the best of our knowledge, the endonuclease fingerprint for DNA damage by hydroxyl radical under low-oxygen conditions has not previously been reported. In our experiments, authentic hydroxyl radical was generated by anaerobic γ-radiolysis of N_2O-saturated aqueous buffer.[34] The radiolysis experiments contained all components of the NADPH:cytochrome P450 oxidoreductase assay system (minus tirapazamine) so that comparison to the endonuclease fingerprint for tirapazamine activated by this enzyme system would be possible. We find that treatment of irradiated DNA with exo III, endo IV, endo III, and FPG yields an endonuclease fingerprint similar to that described above

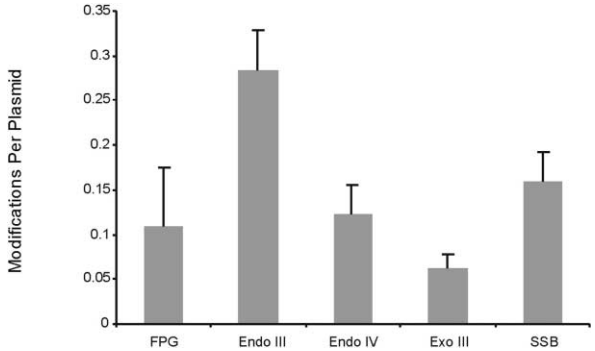


Figure 3. Endonuclease fingerprint for tirapazamine-mediated DNA damage (activated by xanthine oxidase under hypoxic conditions). The reactions utilizing the xanthine/xanthine oxidase enzyme system were performed in the same manner as described for Figure 2, except the assays contained 500 μM xanthine (added to the assay as 3 μL of a 5 mM solution in pH 11 aq NaOH) and 0.4 units/mL xanthine oxidase (Boehringer) in place of NADPH:cytochrome P450 oxidoreductase and the mixture incubated for 1 h prior to precipitation of the DNA.

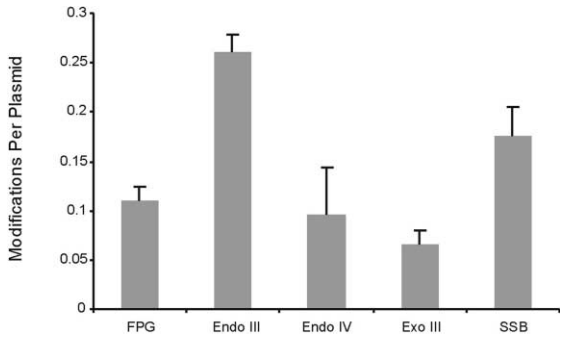


Figure 4. Endonuclease fingerprint for hydroxyl radical under hypoxic conditions. All reactions were performed under nitrous oxide (N_2O). The N_2O used in these experiments contains less than 0.1% O_2. Thus, the final O_2 levels in the radiolysis experiments are comparable to (or below) those found in the enzymatic reactions described here. Nitrous oxide scavenges solvated electrons produced in the radiolysis of water and generates hydroxyl radicals.[34] In the experiment, a solution containing 1 μg pGL2 DNA, 500 μM NADPH, 50 mM sodium phosphate (pH 7.0), 1 mM desferal, 2 mUnits of cytochrome P450 oxidoreductase, and 0.2 mg/mL catalase in a conical-ended Pyrex tube (2 mm ID) was degassed by three freeze-pump-thaw cycles. At the end of the last freeze-pump-thaw cycle, the tube was purged with nitrous oxide (N_2O). The tubes were sealed with rubber septa, the seals wrapped with parafilm, and then exposed to 500 rads of γ-radiation using a ^{60}Co-source. The radiation dose was carefully chosen to maintain levels of supercoiled and nicked plasmid within a range that allows reliable quantitation from the agarose gels (no linearized DNA present). Immediately following irradiation, the DNA was ethanol precipitated as described above and subjected to endonuclease treatment as described in ref 37.

for tirapazamine where oxidized pyrimidine residues (revealed by endo III treatment) are the primary type of enzyme-labile base damage. As a test of our methods, we confirmed that radiolysis of DNA under *aerobic* conditions yields the expected endonuclease fingerprint[23] in which FPG-labile damage predominates over all other types of enzyme-labile damage (data not shown).

The work reported here provides strong evidence that tirapazamine damages the heterocyclic bases in double-stranded DNA under physiologically relevant conditions. DNA base damage can have serious effects on cellular operations and, thus, may contribute to the biological action of this drug.[20–22] The yield of tirapazamine-mediated base damage is significant. The total yield of base damage revealed by FPG and endo III treatment is three to four times greater than the yield of spontaneous strand breaks caused by the drug. The relative amounts of strand cleavage induced by treatment of tirapazamine-damaged DNA by FPG and endo III suggests that tirapazamine inflicts approximately three times more enzyme-labile damage on pyrimidine residues than purine residues in duplex DNA. Initially, the predominance of damage to pyrimidine residues (revealed by endo III treatment) in these experiments may seem surprising because purines (particularly guanine) are more prone to oxidative damage under many conditions.[14] However, the results of our comparison experiments with anaerobic hydroxyl radical indicate that an endonuclease fingerprint showing a predominance of endo III-labile lesions is not unusual for DNA damage by a small, diffusible oxidant under low-oxygen conditions. Indeed, Dizdaroglu's group has previously shown using GC–MS analysis that, under anaerobic conditions, hydroxyl radical causes more damage to pyrimidine residues than to purines.[16,17]

Our finding that tirapazamine causes DNA base damage meshes with the results of previous biological experiments in which Edwards and Virk measured the ability of tirapazamine-damaged phage DNA to infect various strains of repair-deficient *E. coli*.[35] Their results suggested that exo III, uvrC, and endo III were involved in the repair of the tirapazamine-damaged DNA and led to the author's conclusion that single-strand breaks and pyrimidine damage are the major 'inactivating' DNA lesions induced by tirapazamine. It should be noted that our results, along with the earlier results of Edwards and Virk, are inconsistent with the recent proposal that tirapazamine selectively oxidizes the sugar-phosphate backbone of DNA without causing DNA base damage.[13] In that work, ^{32}P-postlabeling analysis successfully detected products of tirapazamine-mediated strand cleavage (e.g., phosphoglycolates), but no evidence for DNA base damage was observed. The reason for the discrepancy between our findings and the results of this previous study is unclear at this time.

Our results show that reductive activation of tirapazamine yields a pattern of DNA damage that is typical of a small, diffusable oxidizing radical (e.g., hydroxyl radical), in which damage to both the DNA backbone and the heterocyclic bases occurs. The exact identity of the base damage inflicted by tirapazamine remains to be determined. Although the endonuclease fingerprint of enzymatically-activated tirapazamine appears similar to that for anaerobic radiolysis (HO•), there are reasons to believe that the drug may yield a distinct spectrum of DNA base lesions. This is because the drug has the ability to *initiate* the formation of DNA radicals and then further *react with* these radical sites in two ways. First, the ability of the drug and its metabolites to transfer oxygen atoms to carbon-centered radicals[11,12] may influence the base damage products. Second, unusual base damage products could result from the reduction of intermediate DNA radicals by the tirapazamine radical anion. To fully understand the biological consequences of tirapazamine-mediated base damage, it will be necessary to characterize the detailed chemical nature of the base damage caused by this drug and to determine whether such damage occurs in vivo. Experiments designed to address these questions are currently underway.

Acknowledgements

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37. Treatment of DNA with Repair Endonucleases. To ensure that all enzyme-labile DNA lesions were converted to cleavage sites, separate aliquots of damaged DNA were treated with increasing amounts of repair enzyme (Trevigen) until the yield of strand breaks reached was seen to reach a maximum. The lowest concentration of each enzyme required to completely convert all DNA lesions into strand breaks was employed in subsequent experiments. The DNA pellets were dissolved 20 μL of the appropriate buffer (Trevigen) containing 2 units of the appropriate repair endonuclease. The solutions were incubated overnight at 37 °C. Following incubation, 10 μL of gel loading buffer was added to each endonuclease reaction and the DNA analyzed by agarose gel electrophoresis as described previously.[7] Values were not corrected for differential ethidium staining of form II and I DNA. The *S*-values (strand breaks per plasmid) were calculated using the equation $S = -\ln(f_1)$, where f_1 is the fraction of plasmid present in the supercoiled form (form I). The values shown in the figures are corrected for the background levels of strand cleavage produced when plasmid DNA that has been subjected to the assay conditions minus drug or minus radiolysis was treated with repair endonucleases. The error bars shown represent the standard error from three measurements.