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. This compound shows clinical promise and is currently being examined in a variety of clinical trials, including several phase III studies. It is important to note that TPZ may be just the first in a new class of hypoxia-selective antitumor agents defined by the heterocyclic N-oxide pharmacophore.8–11

When TPZ12 enters a cell, the drug undergoes enzymic one-electron reduction to yield a radical intermediate that exists as a radical cation. The radical cation then oxidizes the drug along with the unique chemical properties of the drug, yielding a variety of base damages that block DNA transcription and replication. Overall, the results indicate that DNA base damage may contribute to the biological effects of tirapazamine in vivo.

Introduction

In the area of cancer chemotherapy, there is a longstanding desire to identify agents with selective toxicity against cancer cells. The novel antitumor agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, TPZ, Scheme 1) achieves this goal by capitalizing on the unique chemical environment found in tumor cells.1,2 Specifically, TPZ is a bioreductively activated DNA-damaging agent that selectively kills the oxygen-poor (hypoxic) cells found in virtually all solid tumors.1–6 This compound shows great clinical promise and is currently being examined in a variety of clinical trials, including several phase III studies. It is important to note that TPZ may be just the first in a new class of hypoxia-selective antitumor agents defined by the heterocyclic N-oxide pharmacophore.8–11

When TPZ12 enters a cell, the drug undergoes enzymic one-electron reduction to yield a radical intermediate that exists as a radical cation. The radical cation then oxidizes the drug along with the unique chemical properties of the drug, yielding a variety of base damages that block DNA transcription and replication. Overall, the results indicate that DNA base damage may contribute to the biological effects of tirapazamine in vivo.

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a mixture of the radical anion (2a) and the protonated neutral radical (2b, Scheme 1). In normally oxygenated cells, the activated drug (2) is rapidly back-oxidized to starting material (1) by reaction with O₂ (Scheme 1). In the hypoxic environment of tumors, however, the drug radical causes oxidative cleavage of the DNA backbone. The exact nature of the chemical species responsible for TPZ-mediated strand cleavage remains uncertain. It has long been assumed that the one-electron reduction of TPZ leads to production of a highly oxidizing radical species.

Normally, radical-mediated strand breakage in DNA is inefficient under hypoxic conditions. However, reactions with O₂ are required to convert intermediate DNA radicals into strand breaks. Nonetheless, an alternative mechanism has recently been suggested involving dehydrogenation of activated TPZ (2b) to yield the benzo-triazinyl radical 4, which is postulated to be the ultimate DNA-damaging species generated following reductive activation of the drug. While the precise nature of the reactive intermediate(s) responsible for DNA strand cleavage remains controversial, there seems to be general agreement that one-electron reduction of TPZ leads to production of a highly oxidizing radical species.

Scheme 1

![Scheme 1](image)

O₂ to form intermediate DNA radicals into strand breaks. However, recent research has shown that TPZ can play a dual role in DNA strand breakage that allows the drug to overcome this oxygen requirement. In addition to initiating the formation of DNA radicals, TPZ and its metabolites can react with the DNA radicals, resulting in oxygenation of these radical sites. Thus, it appears that the drug may effectively substitute for O₂ in the conversion of DNA radicals into DNA strand breaks and alkali-labile lesions.

It is well established that DNA is an important cellular target for TPZ. Thus, the structural nature of the DNA damage caused by TPZ may ultimately determine many of the cellular responses to this drug. The desire for a more complete understanding of the chemical events underlying the biological activity of this promising antitumor agent provides an incentive to fully characterize the structural nature of the DNA damage caused by TPZ under hypoxic conditions. Analysis of the products resulting from TPZ's biologically relevant reactions will shed light on the chemical mechanisms by which heterocyclic N-oxides effectively deliver cytotoxic DNA-damaging radicals to the interior of hypoxic cells and may ultimately facilitate the development of second generation antitumor agents within this class of drugs.

As part of the effort to understand the chemical events responsible for the hypoxia-selective cytotoxicity of TPZ, it is important to characterize this drug’s ability to damage the genetic information stored in the heterocyclic base residues of double-stranded DNA. It is well-known that damage to DNA bases can have serious biological consequences including mutagenesis, inhibition of gene expression, blockage of DNA replication, and cytotoxicity. While it has long been known that reductive activation of TPZ leads to production of a highly oxidizing radical species.
reactive radical species (possibly *OH) that is able to mediate oxidative damage to the sugar–phosphate backbone in DNA,\textsuperscript{32,16,17,36,52} until lately, it remained uncertain as to whether the drug inflicts damage upon the heterocyclic bases. To address this question, we recently reported the use of base-excision repair enzymes that remove oxidatively damaged base residues from DNA to show that TPZ causes significant amounts of DNA base damage.\textsuperscript{33} In these studies, we found that the yield of base damage is three to four times greater than the yield of spontaneous DNA strand breaks caused by the drug.\textsuperscript{33} While our previous work provided evidence that TPZ damages base residues in double-helical DNA under physiologically relevant conditions, these experiments did not provide any detailed information regarding the chemical structure of the base lesions.

In the work reported here, we have examined the chemistry of TPZ in vitro, under well-defined conditions, to build an improved foundation for understanding the potential of this drug to damage the heterocyclic bases of double-stranded DNA inside hypoxic tumor cells. We employed gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) with the isotope-dilution technique to characterize and quantify oxidative DNA base damage mediated by TPZ. The results show that enzymically activated TPZ causes extensive oxidative damage to the heterogeneousplex bases in duplex DNA and provide the first detailed insight regarding the structural identity and quantities of the base lesions.

**Results**

In this study, we investigated DNA base damage by the antitumor drug TPZ. A previous study reported that TPZ did not cause base damage in DNA when activated with the xanthine/xanthine oxidase (X/XO) enzyme system.\textsuperscript{35} The main damage to DNA observed in this earlier work was determined to be single-strand breaks indicating damage to the sugar moiety. The suggestion that TPZ selectively causes damage to sugar residues in DNA was surprising because enzymic reduction of TPZ clearly leads to production of *OH (or a similarly potent oxidizing agent).\textsuperscript{36} Strong oxidizing agents such as *OH generate a multiplicity of base modifications in DNA including purine- and pyrimidine-derived lesions and 8,5′-cyclopurine-2′-deoxy-nucleosides along with sugar damage that leads to single- and double-strand breaks.\textsuperscript{32,35,34,54–56} The 8,5′-cyclopurine-2′-deoxy-nucleoside lesions are typically formed\textsuperscript{57} by the attack of *OH on DNA under the low O\textsubscript{2} conditions where TPZ is active. Thus, we anticipated that the action of TPZ should yield significant amounts of structurally diverse oxidized DNA base lesions. To test this hypothesis, we employed GC/MS and LC/MS to search for modified bases and 8,5′-cyclopurine-2′-deoxy-nucleosides that might be formed in DNA upon treatment with TPZ in the presence of X/XO under hypoxic conditions. GC/MS and LC/MS are capable of identification and quantification of a multiplicity of modified bases and nucleosides in DNA (for a review, see ref 57).

**DNA Damage Reactions.** As part of these experiments, we have devised a system for inflicting controlled levels of TPZ-induced damage on DNA. In this experimental design, calf thymus DNA and the activating enzyme XO were placed inside a dialysis bag and suspended in a buffered solution containing the enzyme substrate (X) and TPZ. This system serves as a simple model for TPZ-mediated DNA damage in hypoxic cells. It has been suggested that NADPH/cytochrome P450 reductase or a related enzyme is primarily responsible for the in vivo activation of TPZ,\textsuperscript{58,59} but previous studies clearly show that the X/XO enzyme system used here converts TPZ to the same DNA-damaging intermediate\textsuperscript{13,16,17} and yields an identical spectrum of drug metabolites.\textsuperscript{13,16,60,61} 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 enzymic 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 TPZ and X inside the dialysis compartment for extended periods of time, thus, allowing the enzymatic activation of TPZ to proceed efficiently while avoiding product inhibition and scavenging of drug-derived radicals, which sometimes limit the yields of DNA damage that can be achieved for bioreductively activated drugs in vitro.

**Analysis of DNA Base Damage by TPZ.** Using GC/MS to analyze acidic DNA hydrolysates, the following modified bases were identified and quantified in DNA samples: 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 4,6-diamino-5-formamidopyrimidine (FapyAde), 5-hydroxy-6-hydrothymine (5-OH-6-HThy), 5-hydroxy-6-hydrouracil (5-OH-6-HUra), 8-hydroxygenanine (8-OH-Gua), 8-hydroxyadenine (8-OH-Ade), 2-hydroxyadenine (2-OH-Ade), thymine glycol (ThyGly), 5-hydroxyxycytosine (5-OH-Cyt), 5-hydroxyuracil (5-OH-Ura), isodicaric acid [determined as 5,6-dihydroxyuracil (5,6-diOHUra)], and 5-(hydroxymethyl)uracil (5-OHMeUra). It should be noted that the uracil derivatives in DNA result from the deamination of the cytosine derivatives,\textsuperscript{62} except for 5-OHMeUra, which is a thymine product.\textsuperscript{32,54} The structures of these compounds are

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\text{sigmann, J. M. Biochemistry 2002. 41, 914–921.}
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\[(\text{45})\text{ Duarte, V.; Muller, J. G.; Burrows, C. J. Nucleic Acids Res. 1999. 27, 496–502.}
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\[(\text{54})\text{ von Sonntag, C. The Chemical Basis of Radiation Biology; Taylor and Francis: London, 1987.}
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\[(\text{56})\text{ Burrows, C. J.; Muller, J. G. Chem. Res. 1998. 98, 1109–1151.}
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\[(\text{60})\text{ Laderoute, K.; Rauth, A. M. Biochem. Pharmacol. 1986. 35, 3417–3420.}
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illustrated in Figure 1. The levels of DNA base lesions measured in this work are shown in Table 1. Exposure of DNA to the TPZ/X/XO system significantly increased the levels of all the modified bases except 8-OH-Gua and 5-OHMeUra over those observed in control samples. The lack of 8-OH-Gua formation is remarkable, because, in general, this compound is one of the major products formed in DNA by $\cdot$OH reactions.56,57 To confirm this observation, we also used formamidopyrimidine glycosylase (Fpg) instead of acidic hydrolysis to remove 8-OH-Gua from DNA prior to GC/MS analysis because of the potential but avoidable artifactual formation of this compound from free guanine during derivatization of acid-hydrolysates of DNA.63,64 Fpg does not release intact guanine, thus avoiding the formation of 8-OH-Gua from free guanine. Furthermore, we applied LC/MS following enzymic hydrolysis of DNA samples to measure the nucleoside form of 8-OH-Gua, that is, 8-hydroxy-2′-deoxyguanosine (8-OH-dGuo) using a recently developed methodology.65 As Figure 2 illustrates, the levels of 8-OH-Gua measured by GC/MS following Fpg hydrolysis or by LC/MS confirmed those obtained by GC/MS following acidic hydrolysis. These results clearly indicate that 8-OH-Gua was not formed in significant amounts by the TPZ/X/XO system. The use of Fpg hydrolysis also confirmed the formation of FapyGua and FapyAde that was initially seen by acidic hydrolysis (Table 1).

It should be noted that the levels of these compounds measured using Fpg hydrolysis are lower than those measured using acidic hydrolysis. It is known that Fpg does not completely excise its substrates from DNA when they are present at high levels as is the case in this work.62,66

Formation of 8,5′-Cyclopurine-2′-deoxyribonucleosides. Exposure of aqueous solutions of DNA to ionizing radiation under anoxic conditions generates 8,5′-cyclopurine-2′-deoxyribonucleosides.63,64

Table 1. Modified DNA Bases and Their Yields (Lesions/10^6 DNA Bases) as Measured by GC/MS Following Acidic Hydrolysis

<table>
<thead>
<tr>
<th>Product</th>
<th>Control</th>
<th>XXO</th>
<th>TPZ</th>
<th>TPZ/X/XO</th>
</tr>
</thead>
<tbody>
<tr>
<td>FapyGua</td>
<td>9.5 ± 1.7</td>
<td>8.1 ± 1.4</td>
<td>11.1 ± 2.3</td>
<td>1157 ± 95 (564 ± 78)</td>
</tr>
<tr>
<td>5-OH-6-Thy</td>
<td>6.6 ± 1.2</td>
<td>5.3 ± 1.0</td>
<td>6.4 ± 2.8</td>
<td>850 ± 122</td>
</tr>
<tr>
<td>5-OH-6-HUra</td>
<td>27.8 ± 4.0</td>
<td>27.0 ± 4.5</td>
<td>17.1 ± 2.7</td>
<td>617 ± 23</td>
</tr>
<tr>
<td>FapyAde</td>
<td>7.0 ± 1.0</td>
<td>7.1 ± 0.4</td>
<td>5.6 ± 2.1</td>
<td>279 ± 9 (49.3 ± 7.8)</td>
</tr>
<tr>
<td>8-OH-Ade</td>
<td>21.2 ± 5.0</td>
<td>70.8 ± 13.3</td>
<td>20.0 ± 3.8</td>
<td>237 ± 18</td>
</tr>
<tr>
<td>5-OH-Ura</td>
<td>33.1 ± 11.3</td>
<td>33.7 ± 8.1</td>
<td>24.9 ± 1.9</td>
<td>124 ± 23</td>
</tr>
<tr>
<td>5-OH-Cyt</td>
<td>41.2 ± 4.0</td>
<td>44.7 ± 14</td>
<td>34.7 ± 7.4</td>
<td>114 ± 24</td>
</tr>
<tr>
<td>5,6-diOHUra</td>
<td>48.1 ± 10.4</td>
<td>105 ± 7.5</td>
<td>41.3 ± 0.2</td>
<td>114 ± 19</td>
</tr>
<tr>
<td>2-OH-Ade</td>
<td>11.1 ± 8.4</td>
<td>58.3 ± 10.3</td>
<td>11.0 ± 0.7</td>
<td>103 ± 7</td>
</tr>
<tr>
<td>ThyGly</td>
<td>9.2 ± 1.3</td>
<td>8.5 ± 2.5</td>
<td>3.9 ± 0.7</td>
<td>53.7 ± 4.3</td>
</tr>
<tr>
<td>8-OH-Gua</td>
<td>25.4 ± 4.0</td>
<td>20.7 ± 4.1</td>
<td>22.6 ± 4.8</td>
<td>27.6 ± 2.6 (29.9 ± 3.3)</td>
</tr>
<tr>
<td>5-OHMeUra</td>
<td>6.1 ± 2.2</td>
<td>8.5 ± 2.8</td>
<td>6.5 ± 2.3</td>
<td>11.3 ± 1.0</td>
</tr>
</tbody>
</table>

*a Values represent the mean standard deviation of three independent measurements. The values were obtained following hydrolysis of DNA by Fpg.

Figure 1. Structures of the compounds identified and quantified in this work as products of reactions of TPZ with DNA.
sides via a mechanism involving abstraction of a C5′--H atom from deoxyribose by ·OH, followed by cyclization of the C5′-sugar radical onto the C8-position of the purine and, finally, oxidation of the resulting nucleobase radical (reviewed in ref 55). This reaction is inhibited under aerobic conditions, because the diffusion-controlled reaction of O2 with the carbon-centered sugar radical competes with cyclization. The fact that activated TPZ abstracts hydrogen atoms from the deoxyribose backbone of DNA under hypoxic conditions13,16,17 led us to suspect that 8,5′-cyclopurine-2′-deoxynucleosides might be formed by this drug. To test this hypothesis, we analyzed aliquots of DNA samples by GC/MS and LC/MS following enzymic hydrolysis of DNA to nucleosides as recently described.67,68 (5′R)-8,5′-cdGuo, (5′S)-8,5′-cdGuo, (5′R)-8,5′-cdAdo, and (5′S)-8,5′-cdAdo were identified and quantified in DNA samples. As observed previously, background levels of (5′S)-8,5′-cdGuo are higher than those for the (5′R)-isomer.67 Figures 3 and 4 illustrate the levels of these compounds. GC/MS and LC/MS yielded similar results. The results illustrated in Figures 3 and 4 clearly show that (5′R)-8,5′-cdGuo, (5′S)-8,5′-cdGuo, (5′R)-8,5′-cdAdo and (5′S)-8,5′-cdAdo were produced in DNA by the TPZ/X/XO system. This is the first observation of the formation of these compounds by a DNA-damaging agent other than ionizing radiation. For comparison, we also analyzed DNA samples that were exposed to ·OH generated by exposure of N2O-saturated aqueous buffered solution to ionizing radiation. This allowed us to compare the yields of each 8,5′-cyclopurine-2′-deoxy- nucleoside diastereomer formed by radiation-generated ·OH and the TPZ/X/XO system. Figure 3 shows that the amount of (5′R)-8,5′-cdGuo produced by ionizing radiation was similar to that produced by TPZ/X/XO. In contrast, ionizing radiation generated 2 times more (5′S)-8,5′-cdGuo than did TPZ/X/XO. In both cases, the (5′S)-diastereomer was produced more than the (5′R)-diastereomer, in agreement with recently published data.67 The ratios of (5′R)-8,5′-cdGuo to (5′S)-8,5′-cdGuo were 0.5 for TPZ/X/XO and 0.24 for ionizing radiation. The results show a surprising difference in the stereoselectivity of the damage done by these two different DNA-damaging systems. A similar but more dramatic trend was observed for the diastereomers of 8,5′-cdAdo (Figure 4). The ratios of the (5′R)-8,5′-cdAdo to (5′S)-8,5′-cdAdo were 77 for TPZ/X/XO and 3 for ionizing radiation. This means that TPZ/X/XO almost exclusively produced the (5′R)-diastereomer of 8,5′-cdAdo, whereas ionizing radiation generated significant amounts of both the (5′R)- and (5′S)-diastereomers.

**Discussion**

Our results provide the first direct evidence that the antitumor agent TPZ causes extensive enzyme-activated oxidative damage to the heterocyclic bases of DNA under hypoxic conditions. The current results are consistent with our previous studies which indicate that tirapazamine causes more base damage than direct strand breaks.53 A variety of base lesions and tandem 8,5′-cyclopurine-2′-deoxynucleoside lesions were produced. We observed extensive formation of products such as 5-OH-6-HThy, 5-OH-6-HUra, and 8,5′-cyclopurine-2′-deoxynucleosides that are typical for the reaction of ·OH with DNA under anoxic conditions.32,55 Thus, our data are consistent with the previous suggestion that ·OH plays a role in TPZ-mediated DNA damage.17 The major products identified in TPZ-damaged DNA showed an unusual predominance of FapyGua, 5-OH-6-HThy, 5-OH-6-HUra, and FapyAdo relative to the spectrum of base damage typically seen69 for radiolytically generated ·OH under anaerobic or reducing conditions. This is quite noteworthy when one considers the mechanism by which these lesions may be formed. In the case of ionizing radiation, these compounds typically result from reduction of intermediate OH-adduct radicals formed by the addition of ·OH to guanine, thymine,
cytosine, and adenine, respectively (Scheme 2).\textsuperscript{32,55,56,70–73} Similarly, in our case, it is most straightforward to envision that the major products are formed by a mechanism involving initial addition of TPZ-derived OH to the DNA bases, followed by reduction of the intermediate OH-adduct radicals by the TPZ/X/XO system (Scheme 2). Here, it is important to remember that reductively activated TPZ exists as a mixture of the protonated and unprotonated forms in the physiological pH range (the pK\textsubscript{a} of 2b is \textasciitilde 6.0).\textsuperscript{13,15,74} The radical anion of the drug (2a) acts as a reducing agent (e.g., it is easily oxidized by O\textsubscript{2}; the reduction potential of TPZ is \textasciitilde 0.45 V vs NHE at pH 7), while reactions stemming from the protonated form (2b) are responsible for the oxidizing properties of the drug.\textsuperscript{13,15,74} The efficient reduction of OH-adduct radicals by the radical anion of the drug (2a) in this DNA-damage process may be facilitated because, under low-oxygen conditions where the drug operates, alternate reactions involving the combination of base radicals with O\textsubscript{2} are not efficient. It must be noted that, at this time, it is not possible to rule out more complex mechanisms for the initial stages of these base damage reactions involving either formation of intermediate TPZ-base adducts or one-electron oxidation of the bases by activated TPZ (2 or 4), followed by addition of HO\textsuperscript{−} to the resulting radical cations. It has previously been proposed that activated tirapazamine may damage DNA via direct hydrogen atom abstraction;\textsuperscript{1,12,18,29} however, it does not seem likely that direct hydrogen atom abstraction by species such as 2 or 4 could lead to most of the base damage products observed in this study.

The absence of 8-OH-Gua formation is quite remarkable in that various DNA-damaging agents generally produce this compound as one of the major products in DNA.\textsuperscript{55,56} We used three different methodologies to confirm that this compound was not formed by TPZ/X/XO. GC/MS using acidic hydrolysis or Fpg hydrolysis and LC/MS using enzymic hydrolysis provided similar results. This clearly established that 8-OH-Gua is not produced by TPZ under our experimental conditions. Typically, 8-OH-Gua and FapyGua result, respectively, from one-electron oxidation and one-electron reduction of the OH-adduct radical formed upon addition of OH to the C8-position of guanine (Scheme 2).\textsuperscript{55,56,70,73} The absence of 8-OH-Gua formation suggests that the one-electron oxidation of the C8−OH-adduct radical of guanine does not take place in the presence of TPZ/X/XO to a significant extent. Rather, the C8−OH-adduct radical of guanine may be exclusively reduced by TPZ/X/XO to yield FapyGua. Alternatively, it is possible that 8-OH-Gua formed by TPZ undergoes further oxidation under the reaction conditions. It has been reported that 8-OH-Gua is readily oxidized.\textsuperscript{76,77} In contrast to the results observed for guanine, FapyAde and 8-OH-Ade were formed in similar yields. This indicates that the C8−OH-adduct radical of adenine can undergo either oxidation or reduction under the reaction conditions. The differences between adenine and guanine radicals in terms of product formation might be due to their different reduction potentials.\textsuperscript{78} Other base lesions identified in TPZ-damaged DNA result from oxidation of intermediate radicals produced by the addition of *OH to DNA bases, with the exception of 5-OHMeUra, which is formed by oxidation of the allyl radical of thymine.\textsuperscript{32,55} Although the major DNA base damage produced by TPZ/X/XO arises from reduction of OH-adduct radicals, the other damaged bases detected in this study indicate that oxidation of OH-adduct radicals can occur to a reasonable extent in the presence of the TPZ/X/XO system.

TPZ produces substantial yields of the tandem lesions 8,5′-cyclopurine-2′-deoxynucleosides. This is the first observation of the formation of these compounds by a DNA-damaging agent other than ionizing radiation. These lesions are typically formed by hydrogen atom abstraction from the C5′-position of the 2-deoxyribose moiety, followed by attack of the sugar radical on the C8-position of the purine ring and subsequent oxidation of the resulting N-centered purine radical.\textsuperscript{55} Formation of the 8,5′-cyclopurine-2′-deoxynucleosides occurs preferentially under low-oxygen conditions, such as those employed in the current studies. This is because the cyclization step is inhibited by the diffusion-controlled trapping of the C5′-sugar radical by O\textsubscript{2}. There are two diastereoisomers possible for the 8,5′-cyclopurine-2′-deoxynucleoside lesions. Interestingly, TPZ/X/XO and radiation-generated *OH produce substantially different ratios of the two diastereomers. TPZ/X/XO almost exclusively produced (5′R)-8,5′-cdAdo (77:1 ratio), whereas the ratio of the yields of (5′R)- and (5′S)-8,5′-cdAdo was approximately 3 in DNA treated with ionizing radiation. In the case of guanine, the ratios of the (5′R)-8,5′-cdGua to (5′S)-8,5′-cdGua were 0.5 for TPZ/X/XO and 0.24 for ionizing radiation. The reasons why TPZ/X/XO and ionizing radiation produce different ratios of 8,5′-cyclopurine-2′-deoxynucleoside diastereomers is unclear. It is unlikely that TPZ alters the stereochemistry of the cyclization process by distorting the structure of the DNA because the drug does

(70) Steenken, S. Chem. Rev. 1989, 89, 503−520.
not bind noncovalently with the double helix. The differences in the stereochemistry may indicate that TPZ and ionizing radiation produce the 8,5′-cyclopurine-2′-deoxynucleosides by distinct reaction mechanisms. It is possible that the drug’s expected ability to oxygenate the C5-deoxypyridine radical affords a novel chemical route for the formation of 8,5′-cyclopurine-2′-deoxynucleosides. This possibility may be examined in future studies.

It is well-known that oxidative damage to DNA bases can have serious biological consequences including mutagenesis, blockage of DNA replication, inhibition of gene expression, and cytotoxicity (reviewed in ref 47). Among the pyrimidine lesions identified in this study, 5-OH-Cyt and 5-OH-Ura are strongly cytotoxic, leading to C → T transitions. However, the mutagenicity or lethality of these lesions in vivo is not yet known. The other major purine lesion detected in these studies, 8-OH-Ade, is not cytotoxic or mutagenic in bacteria. However, this lesion causes A → G and A → C mutations in mammalian cells. The purine lesion 2-OH-Ade exhibits no cytotoxicity but causes A → G mutations in mammalian cells. Finally, 8-OH-Gua is highly mutagenic (reviewed in ref 84) but was not produced by TPZ/X/XO in any detectable amount under the experimental conditions used in the present study.

A number of the base lesions detected in this work may contribute to the medicinally relevant cytotoxic properties of TPZ. ThyGly is not thought to be mutagenic but blocks polymerases and is considered a lethal lesion. If 5-OH-6-HThy behaves in a manner analogous to ThyGly, it is expected that this would be a cytotoxic lesion. Consistent with this notion, computational and experimental studies provided evidence that 5-OH-6-HThy inhibits polymerases by disrupting base stacking within DNA. By inference, 5-OH-6-HUra might also be a block to polymerases. Thus, two of the primary pyrimidine lesions produced by TPZ/X/XO, 5-OH-6-HThy, and 5-OH-6-HUra, along with the minor lesion ThyGly, are expected to contribute to the cell-killing effects of TPZ.

The 8,5′-cyclopurine-2′-deoxynucleosides produced by the action of TPZ also appear to be cytotoxic lesions. This conclusion is based on the results of recent studies on 8,5′-cdAdo. Both (5′R)- and (5′S)-diastereomers of this compound block primer extension by mammalian and microbial DNA polymerases. (5′S)-8,5′-cdAdo was also found to be a blocker of gene expression in CHO and human cells. These studies indicate that (5′R)-8,5′-cdAdo and (5′S)-8,5′-cdAdo are potentially cytotoxic lesions. Moreover, recent data obtained using human DNA polymerase η and 8,5′-cdAdo-containing oligonucleotides showed that the (5′S)-diastereomer might be more cytotoxic than the (5′R)-diastereomer in vivo. Although no data exist on the biological effects of 8,5′-cdGuo, one can assume by inference that this lesion possesses cytotoxic properties similar to 8,5′-cdAdo. We find that the yield of 8,5′-cdGuo produced by TPZ/X/XO was significantly greater than that of 8,5′-cdAdo. The putatively more toxic (5′S)-8,5′-cdGuo was produced in a greater yield than (5′R)-8,5′-cdGuo. In the case of the 8,5′-cdAdo, the less toxic (5′R)-diastereomer was formed preferentially over the (5′S)-diastereomer by the action of TPZ/X/XO on DNA. These findings suggest that 8,5′-cdGuo may contribute to the drug’s cytotoxicity more than 8,5′-cdAdo. Overall, the data in this study, along with previously published work, strongly suggest that 8,5′-cyclopurine-2′-deoxynucleosides could play a significant role in the biological effects of TPZ in vivo.

Finally, the observed DNA base damage may provide a framework for understanding the reported involvement of topoisomerases in the cytotoxicity of tirapazamine. Although tirapazamine and its metabolites are not direct-acting topoisomerase inhibitors, it has been reported that exposure of cells to the drug under hypoxic conditions leads to inhibition of topoisomerase II. We suggest that cellular attempts to repair TPZ-mediated base damage could lead to inhibition of topoisomerases. This idea is supported by recent studies showing that abasic sites formed in DNA during base excision repair can serve as topoisomerase poisons and may lead to double-strand breaks. Most of the DNA base lesions detected in the current work are known substrates for base-excision repair enzymes. Thus, to the extent that the abasic intermediates produced during repair of these damaged bases are accessible to DNA topoisomerases, oxidative base damage may provide an explanation for the reported tirapazamine-mediated inhibition of topoisomerases.

In summary, we have provided the first structural characterization of DNA base damage by the promising new antitumor agent TPZ. The unique variety of DNA base lesions produced by this drug may contribute to its medicinally useful biological properties. Studies are underway to better understand how the spectrum of TPZ-mediated base damage responds to various physiologically relevant changes in reaction conditions. Ultimately, it will be important to extend these studies to the characterization of DNA base damage produced by this drug in vivo.
Experimental Section

Materials. TPZ was prepared as described previously. Modified DNA bases, their stable isotope-labeled analogues, and other materials for GC/MS were obtained as described previously. Nucleoside P1, phosphodiesterase I, phosphodiesterase II, xanthine (X), and xanthine oxidase (XO) were obtained from Sigma Chemical Company (St. Louis, MO). Alkaline phosphatase was purchased from Roche Diagnostics Corporation (Indianapolis, IN). 2′-Deoxycytidine-5′-triphosphate-1,3,7,9-15N4(2-amino-15N) (dGTP-15N) and 2′-deoxyadenosine-5′-triphosphate-1,3,7,9-15N4(4-amino-15N) (dATP-15N) were purchased from Medical Isotopes, Inc. (Pelham, NH). (5′S)-8,5-cyclo-2′-Deoxyadenosine ([5′S]-8,5-c’dAdo) was obtained from Berry & Associates, Inc. (Ann Arbor, MI). Acetonitrile (HPLC grade) was from Burdick and Jackson (Muskegon, MI). Biomax5 ultra filtration membranes (molecular mass cutoff of 5 kDa) were purchased from Millipore (Bedford, MA). Water (HPLC-grade) for LC/MS analyses was from J. T. Baker (Phillipsburg, NJ). Water purified through a Milli-Q system (Millipore, Bedford, MA) was used for all other applications. Formamidopyrimidine glycosylase (Fpg) was kindly provided by Dr. Tim Connor of the City of Hope Medical Center (Duarte, CA). Certain commercial equipment or materials or equipment identified are necessarily the best available for the purpose.

Preparation of Stable Isotope-Labeled Analogues of 8,5-cyclo-2′-Deoxyadenosine and 8,5′-cyclo-2′-Deoxyguanosine. Stable isotope-labeled analogues of the (5′R)- and (5′S)-diastereoisomers of 8,5′-cyclo-2′-deoxyadenosine (8,5′-c’dGuo) were prepared as described. Stable isotope-labeled analogues of the (5′R)- and (5′S)-diastereoisomers of 8,5′-cdAdo were prepared in a similar fashion using commercially available dATP-15N. An N2O-saturated aqueous solution of dATP-15N5 (5 mg/100 mL) was exposed to ionizing radiation in a 60Co γ-source at a dose of 400 Gy (30 Gy/min) as described. This treatment was expected to produce both (5′R)-8,5′-cdATP-15N5 and (5′S)-8,5′-cdATP-15N5 from dATP-15N5 among other products, because it is well-known that the exposure of oxygen-free aqueous solutions of dAdo to ionizing radiation generates (5′R)-8,5′-cdAdo and (5′S)-8,5′-cdAdo. Irradiated samples of dATP-15N were dephosphorylated and filtered as described. An aliquot (5 μL) of the dephosphorylated and filtered sample was analyzed by LC/MS and found to contain both (5′R)-8,5′-cdAdo-15N5 and (5′S)-8,5′-cdAdo-15N5 on the basis of the previously reported LC/MS analysis of (5′R)-8,5′-cdAdo and (5′S)-8,5′-cdAdo. Another aliquot was lyophilized, trimethylsilylated, and analyzed by GC/MS. This analysis also confirmed the presence of these compounds on the basis of the previously reported mass spectral characteristics of the trimethylsilyl derivatives of (5′R)-8,5′-cdAdo and (5′S)-8,5′-cdAdo. Subsequently, semipreparative LC was used to isolate (5′R)-8,5′-cdAdo-15N5 and (5′S)-8,5′-cdAdo-15N5 eluted at 11.8 and 16.6 min, respectively, and were completely separated from cdAdo-15N5, which eluted at 17.5 min. The fractions corresponding to (5′R)-8,5′-cdAdo-15N5 and (5′S)-8,5′-cdAdo-15N5 were collected. At least 50 injections of 100 μL aliquots were performed. Collected fractions were dried in a SpeedVac under vacuum and then dissolved in 200 μL of water. The absorption spectra of the solutions of (5′R)-8,5′-cdAdo and (5′S)-8,5′-cdAdo were recorded between the wavelengths of 210 and 350 nm. The spectra were identical to the absorption spectrum of authentic (5′S)-8,5′-cdAdo, which was commercially available. The identity and purity of the compounds were checked and confirmed by LC/MS using the analytical column. The isolated compounds were pure and did not contain any detectable unlabeled analogues. Their positive-ion mass spectra consisted of the protonated molecular ion at m/z 255 and an ion at m/z 169 due to cleavage of the N-glycosidic bond and the bond between the 5′-carbon and 4′-carbon of the sugar moiety, as expected on the basis of the previously published mass spectra of (5′R)-8,5′-cdAdo and (5′S)-8,5′-cdAdo. The concentrations of the solutions of (5′R)-8,5′-cdAdo-15N5 and (5′S)-8,5′-cdAdo-15N5 were 0.085 mM and 0.035 mM, respectively, as determined by UV spectrosopy using the absorption coefficient of 15 200 M−1 cm−1 at 265 nm and by LC/MS using an aliquot of 0.1 mM solution of (5′S)-8,5′-cdAdo as an internal standard.

Treatment of DNA with TPZ. In a typical assay, 250 μL of a solution containing highly polymerized calf thymus DNA (60 μg), XO (100 μU), catalase (100 μg/mL), SOD (10 μg/mL), desferal (1 mM), and sodium phosphate buffer (10 mM, pH 7.0) was prepared. Individual components of this solution (except for DNA and enzymes) were degassed by three cycles of freeze–pump–thaw in Pyrex tubes and then torchsealed under vacuum. The sealed tubes were stored, transferred to a glovebag filled with argon, opened, and then used to prepare the solution. This solution was transferred to a slide-a-lyzer minidialysis unit (MW cutoff 3500; Pierce cat #909595), and the unit floated atop 5 mL of a freeze–pump–thaw degassed solution containing TPZ (250 μM), X (500 μM), desferal (1 mM), and sodium phosphate buffer (10 mM, pH 7.0). The apparatus was gently stirred inside the argon-filled glovebag for 16 h at 24 °C. The DNA-containing solution was then dialyzed for 24 h twice against 5 mL of sodium phosphate buffer (10 mM, pH 7.0) to remove the small molecules xantine, uric acid, desferal, TPZ, and its metabolites. The DNA-containing solution was then removed from the dialysis unit and concentrated in a SpeedVac under vacuum to a final volume of ~100 μL. Finally, the concentration of DNA in the sample was determined by UV spectrosopy. All solutions were prepared with water that was passed through a column of chelex resin to remove trace metals.

Irradiation of DNA. An aqueous buffered solution of DNA (10 mM phosphate buffer, pH 7.4, 0.3 mg/mL) was saturated with N2O and irradiated with γ-rays in an 60Co γ-source at a dose of 20 Gy (dose rate 30 Gy/min). Subsequently, the DNA solution was dialyzed against 10 mM phosphate buffer (pH 7.4) for 18 h. Phosphate buffer outside the dialysis tubes was changed 3 times during the course of dialysis. Aliquots of 50 μg of DNA were dried in a SpeedVac under vacuum.

Acidic and Enzymic Hydrolyses of DNA. Aliquots of stable isotope-labeled analogues of modified bases and dGuo-15N were added to aliquots of 50 μg of DNA samples. dGuo-15N is used to determine the DNA amount by GC/MS. The acidic hydrolysis of this compound yields guanine-15N5, which is used as an internal standard for quantification of guanine in DNA and, thus, the DNA amount. DNA samples were dried under vacuum in a SpeedVac and hydrolyzed with 0.5 mL of 60% formic acid in evacuated and sealed tubes at 140 °C for 30 min. Hydrolysates were lyophilized for 18 h.

For hydrolysis with Fpg, aliquots of DNA samples (50 μg) were dissolved in 100 μL of an incubation buffer consisting of phosphate buffer (final concentration 50 mM, pH 7.4), 100 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol. Samples were incubated with 1 μg of Fpg for 30 min. Aliquots of stable isotope-labeled analogues of modified bases were added to the samples followed by filtration using ultrafiltration membranes with a molecular mass cutoff of 5 kDa by centrifugation at 6000 × g for 30 min. Filtered samples were lyophilized for 18 h.

For enzymic hydrolysis of DNA to nucleosides, aliquots of (5'R)-8,5'S-cdAdo-15N5, (5'S)-8,5'S-cdAdo-15N5, (5'R)-8,5'S-cdGuo-15N5, and (5'S)-8,5'S-cdGuo-15N5 were added to aliquots of 50 µg of DNA samples. This was followed by drying the samples in a SpeedVac under vacuum. DNA samples were dissolved in 100 µL of a 20 mM succinic acid solution (pH 6.0) containing 10 mM CaCl2. Hydrolysis using four enzymes was performed as described previously.7 Hydrolyzed samples were filtered as described above. Six samples were prepared for each data point. Three of the samples were lyophilized for 18 h to be used for GC/MS analysis. The other three samples were used for LC/MS analysis without further treatment.67

Analyses by LC/MS and GC/MS. Analyses by LC/MS with isotope-dilution technique and selected-ion monitoring were performed as previously described.67 Aliquots (30 µL) of filtered enzymic hydrolysates of DNA samples were injected on the column without any further treatment. Measurements by GC/MS with isotope-dilution technique and selected-ion monitoring were performed as described.67 Lyophilized acidic or enzymic hydrolysates of DNA samples were trimethylsilylated with 60 µL of a mixture of bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylchlorosilane) and pyridine (1/1, v/v) at 120 °C for 30 min. Aliquots of 2 µL of the derivatized samples were injected onto the GC column using the split mode of injection with a split ratio of 20 to 1.

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