METABOLICALLY ACTIVATED HETEROCYCLIC N-OXIDE COMPOUNDS FOR KILLING AND VISUALIZING HYPOXIC CANCER CELLS

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Doctor of Philosophy

By
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December 2009
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**METABOLICALLY ACTIVATED HETEROCYCLIC N-OXIDE COMPOUNDS FOR KILLING AND VISUALIZING HYPOXIC CANCER CELLS**

Presented by **Ujjal Sarkar**

A candidate for the degree of Doctor of Philosophy

and hereby certify that in their opinion it is worthy of acceptance

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__________________________________________________________________________

Professor Frank J. Schmidt
I dedicate my thesis work to my wonderful family..........................

I am forever indebted to my parents, Umesh C. Sarkar and Manorama Sarkar for their constant love and encouragement. I sincerely thank them for their sacrifice for my career growth. I would also like to thank my elder brother Utpal Sarkar, elder sister Mithu Chatterjee, and their families for their love and support.

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In my mother tongue “Bengali” I would say “Ami Tomader Bhalo Bashi”.

“Other things may change us, but we start and end with family.”

Anthony Brandt
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ABSTRACT

Tirapazamine (TPZ) is currently undergoing a variety of phase I, II, and III clinical trials for the treatment of various human cancers. TPZ derives its medicinal activity by inducing DNA damage in poorly oxygenated tumor cells. Selective bioreductive enzymatic metabolism of TPZ in tumor cells leads to radical intermediates, which primarily contribute oxidative DNA damage. The nature of radical intermediates responsible for DNA damage is still a matter of debate. At the same time, there is an ongoing effort to prepare TPZ analogues as potential new antitumor agents. Thus, there is immediate need for the development of synthetic methods for the preparation of TPZ analogues.

The very first part of this dissertation provides the utility of Suzuki coupling in the synthesis of 3-alkyl and 3-aryl derivatives of the antitumor agent TPZ. In these studies, the bromo substrate provided improved yields than chloro. To the best of our knowledge, we have provided general scope of Suzuki coupling reaction on the benzotriazine-1-oxide substrates involving various 3-aryl, and 3-cyclopropyl boronic acid to build a series of TPZ analogues.

In addition to this work, we prepared novel 3-cyclopropyl-1,2,4-benzotriazine 1,4-dioxide which damages DNA under bioreductive hypoxic conditions. We also, utilized another 3-alkyl derivative of TPZ, 3-methyl-1,2,4-benzotriazine-di-N-oxide, to reinvestigate the mechanism of TPZ action. Our data imply the release of hydroxyl radical from activated TPZ is a reasonable mechanism to explain the DNA damage. This
information is critical to our understanding of the effect of anticancer agent TPZ on various solid tumors.

We also show for the first time that other class of heterocyclic $N$-oxides such as natural product myxin and methylmyxin behave like redox activated hypoxia selective DNA damaging agent tirapazamine.

In the last part of this thesis, we have explored for the first time the chemistry of the benzotriazine scaffold as a hypoxia-selective fluorescent probe. We have studied with a series of known benzotriazine compounds, and have found a few with a moderate fluorescence quantum yield and molar extinction coefficient. Our novel effort toward hypoxia directed fluorescent small molecule probes may be useful for imaging in cancer therapy, and other hypoxia related diseases.
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Chapter 1

Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine

1.1 Introduction

The compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, TPZ, 4) and other 1,2,4-benzotriazine 1,4-dioxides define a new class of hypoxia selective anticancer agents.1-3 This class of compound undergoes one electron enzymatic reduction to generate radical intermediate that selectively releases a cell killing reactive intermediate under very low oxygen concentrations found inside solid tumors.3 The efficacy of TPZ has been examined in a wide variety of clinical trials; however, TPZ has not yet received FDA approval for use in the treatment of human cancers.4 Accordingly, there is an ongoing effort to prepare TPZ analogues as potential new antitumor agents.5-11
Thus, there is immediate need for the development of synthetic methods for the preparation of TPZ analogues.

1.2 Various Synthesis Methods of TPZ and its Analogues.

The 1,2,4-benzotriazine 1-oxide core (3) is typically prepared via the condensation of 2-nitroaniline (1) with cyanamide (2), and oxidation yields TPZ (Scheme 1).

Condensation of various 2-nitroaniline derivatives bearing substituents on the benzo ring provides wide varieties of TPZ derivatives. Reaction of 1-fluoro-2-nitrobenzene or 1,2-dinitrobenzene with guanidine base also yields 3. Alternatively, the reaction of benzofuroxan with sodium cyanamide provides direct access to TPZ. Similarly, a variety of TPZ analogues bearing alkyl and aryl substituents on the 3-amino group have been prepared by nucleophilic aromatic substitution of amine (Scheme 1.2).
Recent studies have shown that an exocyclic amine in TPZ can limit its potency. The amine group is a good H-donor and causes low tissue diffusion and solubility. 3-alkyl and 3-aryl-1,2,4-benzotriazine analogues are of special interest because they display hypoxia selective cytotoxicities comparable to TPZ. In addition, these analogues may possess superior pharmacokinetic properties. 3-methyl and 3-phenyl-1,2,4-benzotriazine oxides have been prepared by BF₃-catalyzed cyclization of formazan precursors or PtO₂-catalyzed cyclization of the 2-nitrophenyldiazone of pyruvic acid (Scheme 1.3). The palladium-catalyzed Stille coupling reaction also gives rise to 3-alkyl, aryl, vinyl, and allyl 1,2,4-benzotriazine 1-oxides from 3-chloro-1,2,4-benzotriazine 1-oxide (Scheme 1.2).
1.3 **Synthesis of TPZ Analogues: By Suzuki Coupling Reaction**

The Suzuki coupling reaction is an important complementary reaction to the Stille coupling reaction for the formation of carbon-carbon bonds involving the palladium catalyzed coupling of an aryl halide precursor and an alkyl or aryl boronic acid. Other than a single example, Suzuki coupling has not been explored for the preparation of TPZ analogs. Here, we describe the use of Suzuki coupling in preparation of short series of 3-substituted analogs of triazine-1-oxide. These reactions occur under very mild conditions, and a wide variety of boronic acids substrates are readily available. In addition, the boronic acids used in the Suzuki reaction are more environmentally green than the organostannanes used in the Stille coupling. There is only a single report in which the Suzuki-Miyaura coupling reaction has been used to prepare 3-aryl-1,2,4 benzotriazines.\(^\text{19}\)
Consequently, we set out to explore this reaction to make various 3-aryl and 3-alkyl-1,2,4-benzotriazine 1-oxide.

The mono-N-oxide 3 was prepared by the condensation of cyanamide with 2-nitroaniline.\textsuperscript{12-14} 3-halo-1,2,4-benzotriazines were made from 3 by treatment of sodium nitrite in aqueous sulfuric acid, followed by treatment with phosphorus oxyhalide. The halide substrates were coupled with aryl and alkyl boronic acid with the Suzuki coupling reaction (Scheme 1.4). The reaction in Table 1 was described as general preparation methods unless otherwise stated. In a typical reaction, to a solution of bromo-1,2,4-benzotriazine 1-oxide, boronic acid, potassium phosphate, and tricyclohexylphosphine ligand (10 mol% Pcy\textsubscript{3}) in toluene and water (~ 5: 1) under nitrogen atmosphere, was added Pd(OAc)\textsubscript{2} (5 mol\%). The reaction mixture was refluxed at 90 °C – 95 °C overnight and was then allowed to cool to room temperature. After the reaction was complete as judged by thin layer chromatography, water (5 mL) was added and the mixture was extracted with dichloromethane. The combined organics were dried with sodium sulfate and concentrated under rotovap. Purification of the residue by column chromatography eluted with ethyl acetate and hexanes afforded the desired compounds.

\[ \begin{align*}
\text{O}^- & \quad \text{N} \quad \text{N} \quad \text{X} \\
\text{HO} & \quad \text{HO} & \quad \text{B} & \quad \text{R} \\
\text{Pd(OAc)}\textsubscript{2}, \text{Pcy}3 \\
\text{Toluene, H}_2\text{O}, \text{K}_3\text{PO}_4
\end{align*} \]

\textbf{Scheme 1.4:} Coupling reaction of boronic acids and halide substrates
Under these specified reaction conditions, coupling of the chloro substrate 6 with 4-cyanophenylboronic acid and cyclopropylboronic acid proceed in reasonable yield (Figure 1),

![Chemical Structure](image)

While the analogous reaction with 4-bromophenyl boronic acid, 4-nitrophenylboronic acid, and quinolin-2-ylboronic acid gave low yields (Table 1). Use of the bromo-substrate 7 improved the yields in the cases of 4-bromophenylboronic acid and 4-nitrophenylboronic acid. Generally, aryl bromides are more reactive substrates in Suzuki coupling reactions; however, it is worth noting that use of the bromo substrate 7 with 4-cyanophenylboronic acid and cyclopropyl boronic acid did not improve the yields significantly beyond those obtained with the chloro substrate 6. The coupling involving quinoline-2-ylboronic acid proceeded in very low yield even with the bromo substrate 7.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Yield 19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HO B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HO B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X= Cl</td>
<td>47-50%</td>
</tr>
<tr>
<td></td>
<td>X= Br</td>
<td>50-52%</td>
</tr>
</tbody>
</table>

**Figure 1.1:** Suzuki coupling of cycloalkylboronic acid with 3-halo-1,2,4-benzotriazine 1-oxide
Chapter 1
Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine

In this case, we synthesized the compound using the electron–rich Sphos ligand\(^2\) with improved yield.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Boronic acid derivatives</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X= Cl</td>
<td>(HO)(_2)B(\cdots)Br</td>
<td>trace</td>
</tr>
<tr>
<td>2</td>
<td>X= Br</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>3</td>
<td>X= Cl</td>
<td>(HO)(_2)B(\cdots)NO(_2)</td>
<td>trace</td>
</tr>
<tr>
<td>4</td>
<td>X= Br</td>
<td>21</td>
<td>35%</td>
</tr>
<tr>
<td>5</td>
<td>X= Cl</td>
<td>(HO)(_2)B(\cdots)CN</td>
<td>55%</td>
</tr>
<tr>
<td>6</td>
<td>X= Br</td>
<td>22</td>
<td>60%</td>
</tr>
<tr>
<td>7</td>
<td>X= Cl</td>
<td>(HO)(_2)B(\cdots)</td>
<td>trace</td>
</tr>
<tr>
<td>8</td>
<td>X=Br</td>
<td>23</td>
<td>29%</td>
</tr>
</tbody>
</table>

Table 1.1: Cross coupling reactions with various boronic acids and yields of the reactions

1.4 Crystal Structure of Pd\(_2\) Complex

The column chromatography of the reaction between 6 and cyclopropylboronic acid produced a fraction that, upon evaporation, gave a small amount (~1% yield) of crystals which were characterized by X-ray diffraction. Interestingly, the material proved to be a dinuclear triazine-bridged palladium complex resulting from oxidative addition of 6 to palladium. The carbon and nitrogen atoms of two triazines and the two palladium centers formed a six-membered ring in a boat confirmation in which the nitrogens are \textit{trans} to the phosphine ligands and the carbons are \textit{trans} to the chlorines (Figure 1.2). The structure is completely analogous to the one previously reported of the
addition of 2-bromopyridine to Pd(PPh_3)_4^{22}. In light of previous precedent, it is possible
the di-nuclear palladium complex isolated from our reaction may be capable of catalyze
the coupling reactions.

Figure 1.2: Ortep representation of the crystal structure of dinuclear palladium complex

The mechanism of this reaction typically goes via a catalytic cycle involving three
important steps, namely oxidative addition, transmetallation, and reductive elimination.
The catalytic cycle of this reaction is given in scheme 1.3^{22a}
Chapter 1

Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine

Figure 1.3: Suzuki catalytic cycle
1.5 Conclusion

In this chapter, we have presented the utility of Suzuki coupling in the synthesis of 3-alkyl and 3-aryl derivatives of the antitumor agent tirapazamine. The yields in these cross-coupling reactions (40-60%) are somewhat lower that the yields reported in Stille couplings between 6 and organostannanes \(^{28}\). In these studies, the bromo substrate provided improved yields. To the best of our knowledge, we have provided general scope of Suzuki coupling reaction on the benzotriazine-1-oxide substrates involving various 3-aryl and 3-cyclopropyl boronic acids to build a series of TPZ analogues. This could be a useful method to build various 3-alkyl and 3-aryl 1,4 dioxides, which may display improved bioactivity in preclinical studies. \(^{29}\) Further explorations of C-C and N-C coupling reaction on various benzotriazine 1-oxides, are an ongoing effort.
Chapter 1

Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine

1.6 Material, Method and Data

All chemicals were purchased from the following suppliers and were the highest purity available and were used as received without further purification unless otherwise indicated. Cyanamide, 2-nitroaniline, 2-chloro-perbenzoic acid (mCPBA), phosphorus oxychloride (POCl₃), phosphorus oxybromide (POBr₃), potassium phosphate (K₃PO₄), Pd(OAc)₂ (palladium acetate), tri-cyclohexylphosphine (Pcy₃), 2-dicyclocxyphosphino-2’,6’-dimethoxybiphenyl, and tri-cyclohexylphosphine were purchased from Aldrich Chemical Co., cyclopropyl boronic acid, 4-bromophenyl boronic acid, 4-nitrophenyl boronic acid, 4-cyanophenyl boronic acid, 3-quinoline boronic acid were purchased from Boron Molecular; toluene was purchased from Fisher, silica gel (0.04 – 0.063 mm pore size) for column chromatography (Merck), TLC plates were purchased from Aldrich. NMR spectra were obtained using a Bruker DRX 300 MHz instruments at the University of Missouri-Columbia. High resolution mass spectroscopy was performed at the University of Illinois Urbana-Champaign mass spectrometry facility and low resolution mass spectrometry was performed at the University of Missouri-Columbia. Spectra were obtained in CDCl₃ unless otherwise specified, and were referenced to Me₄Si. Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. Thin-layer chromatography was carried out on thin glass based silica gel plates with visualization of components by UV light (254 nm). Column chromatography was carried out on silica gel (230 - 400 mesh). DCM refers to dichloromethane.
3-Cyclopropyl-1,2,4-benzotriazine 1-oxide (19). The compound 3-chloro-1,2,4-benzotriazine 1-oxide\textsuperscript{1,3} (3c, 60 mg, 0.33 mmol), cyclopropyl boronic acid (1.2 eq. 34 mg), potassium phosphate (203 mg), PC\textsubscript{3} (10 mol %), and Pd(OAc)\textsubscript{2} (5 mol %) were added to a nitrogen purged flask and dissolved in a mixture of toluene (2 mL) and water (100 \(\mu\)L). The mixture was then heated refluxed for 24 h. The reaction mixture was allowed to cool to room temperature, and 5 mL of water added. The mixture was then extracted with dichloromethane, and the combined organic layers dried over Na\textsubscript{2}SO\textsubscript{4} and rotary evaporated under reduced pressure. Purification by column chromatography on silica gel eluted with 5 to 25% ethyl acetate in hexane afforded the desired compound 4a as a pale yellow solid in a 47% yield. Reaction with the bromo analog 3b using analogous conditions gave a 52% yield. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 8.42 (1H, d, 8.4 Hz), 8.02-7.87 (2H, m), 7.6-7.5 (1H, m), 2.34-2.26 (1H, m), 1.36 (2H, m), 1.22 (2H, m), \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}): \(\delta\) 168.3, 147.6, 136.6, 135.4, 131.0, 129.1, 128.4, 128.3, 120.3, 120.1, 16.7, 11.1; HRMS (ESI) m/z calc for C\textsubscript{10}H\textsubscript{10}N\textsubscript{3}O (MH\textsuperscript{+}) 188.0824, found 188.0823.
Chapter 1
Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine
Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine
### Table 1.2: Crystal data and structure refinement for Pd₂ complex.

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Table 1.3. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for 3cypbt0. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

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Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine

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References


Chapter 1

Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine

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19) Hay, M. P.; Denny, W. A. A new and versatile synthesis of 3-alkyl-1,2,4-benzotriazine-1,4-dioxides: preparation of the bioreductive cytotoxins SR4895 and SR4941 *Tetrahedron Lett.* 2002 43 9569 9571

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Chapter 1

Palladium Mediated C-C Bond Formation: Toward the Construction of Analogs of Antitumor Drug Tirapazamine


2.1 Introduction

1,2,4-benzotriazine 1,4 oxides are an increasingly attractive field of study in the area of cancer research. These potent antitumor drug candidates undergo in-vivo bioreduction, leading to selective DNA damage in the low oxygen cells found in tumors. TPZ is the lead compound in this class of hypoxia-selective heterocyclic-N-oxides and is currently undergoing phase III clinical trials. TPZ (1) is bireductively metabolized majorly to the radical anion (4a, Scheme 2.1) that exists in equilibrium with its protonated neutral form (4b). We suggest that protonated neutral TPZ radical spit out a
Chapter 2

Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide

hydroxyl radical as cellular DNA damaging intermediate. TPZ shows some mixed results in phase III clinical trial.\(^2\)

Scheme 2.1: Proposed mechanism of DNA damage by metabolically activated TPZ

In recent years, synthesis of various new TPZ analogues with better diffusion and biological properties are an active program. For example, Hay et al., have shown that replacing the exo-cyclic amine with a non hydrogen donor such as an alkyl group can enhance the cytotoxicities of this class of compounds. 3-methyl and 3-ethyl benzotriazine 1,4 dioixdes display hypoxia selective cytotoxicities comparable to tirapazamine. This alkyl substituted \(N\)-oxide compounds may show better pharmacokinetics and extravascular transport properties which are desirable conditions for an optimum drug candidate.\(^9,10\).

2.2 Ring Opening Rates of Cyclopropyl Radicals

The cyclopropyl radical to allyl free radical rearrangement has always been a fundamentally interesting field to study (Scheme 2.2). Ring opening of various cyclopropyl radicals or radical anions are still under investigation. This rearrangement is critically important for both mechanistic and synthetic purposes.\(^{23,24,25}\) The rapid ring
opening or rearrangement of cyclopropyl radical does happen with a rate constant of 1.2 x 10^9 s\(^{-1}\) at 37 °C \(^{32}\)

Several factors can affect this rate, such as lowering temperature to -24 °C, changed the rate of ring opening to 9.5 x 10^5 s\(^{-1}\). Different substituents on the cyclopropane, such as extended conjugated system can also decrease the rate to around 10^4-10^6 s\(^{-1}\) \(^{33,34}\) (Scheme 2.3). Cylopropyl radical anion can also have some solvent effect, which might inhibit the ring opening reaction. Thus, the ring opening reaction for a radical anion can be governed by charge and spin delocalization. \(^{34a}\)

### 2.3 Goals

Given the activity of the 3-alkyl-derivatives, we thought it might be interesting to test 3-cyclopropyl derivative of TPZ. To learn more about fundamental reactions of substituted cyclopropyl carbinyl radicals we set out to perform biochemical studies of a novel TPZ analog 3-cyclopropyl-1,2,4-benzotriazine 1,4-di-N-oxide (32). More importantly, given the importance of radical intermediates in the bioactivity of this class of compounds, we
felt it would be interesting to explore whether the presence of the cyclopropane ring would “derail” the normal mechanism observed within this class of compounds in a way that would profoundly alter their chemical and biological properties. Specifically, cyclopropyl carbinyl radicals have the potential to undergo ring-opening reactions. This property has been widely used in mechanistic and synthetic studies.\textsuperscript{35,35a-c} However, an interesting aspect of the current work is that the properties of cyclopropyl substituents on radical anions are not well defined.\textsuperscript{35,34}

In this study, we have designed and synthesized 3-cyclopropyl-1,2,4-benzotriazine 1,4-dioxide to investigate the effect of cyclopropyl group in the radical mediated chemistry. In other words, we probe cyclopropyl group as a mechanistic handle to understand the nature of radical intermediate. We report the efficiency and mechanism of DNA cleavage by 32.

In addition to this present study, we report the X-ray crystal structure of 3-cyclopropyl-1,2,4-benzotriazine 1,4-dioxide (32). 3-alkyl-1,2,4-benzotriazine 1,4-di-N-oxide class of compounds have been characterized as a potential antitumor agents. To the best of our knowledge, incorporation of cyclopropyl group on benzotriazine scaffold is not known, and is not characterized as antitumor agent. For the first time, we synthesized compound 32 and characterized by X-ray crystallography. The crystal structure of 32 may contribute to understanding of the chemistry and biology of 3-cyclopropyl-1,2,4-benzotriazine 1,4-dioxide.
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Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide

2.4 Results and Discussion

Compound 32 crystallized in the monoclinic space group P2/c. Atomic coordinates and equivalent isotropic displacement parameters of the non-hydrogen atoms are given in Table 2, bond lengths and bond angles are shown in Tables 3 and 4, respectively, and an ORTEP drawing of 32 is shown in Figure 1.

![Figure 2.1: ORTEP diagrams of 32](image)

![Figure 2.2: Packing diagram of 32](image)

Figure 2.2 shows a diagram of the packing viewed normal to the a-c plane. It can be seen that approximately coplanar molecules form layers along the a-c diagonal with considerable overlap of the aromatic rings.
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2.5 Conformation and Rotational Energy Profile

The cyclopropyl group attached to a benzene ring adopts a bisected conformation, that is, the C–H bond of the cyclopropyl carbon that is attached to the benzene ring is coplanar with the arene; \( \tau = \angle(C_{\text{ortho}} - C_{\text{ipso}} - C_{\text{CP}} - H) = 0^\circ \).\(^{22}\) In the bisected conformation the molecular orbital overlap between the cyclopropyl group and the arene \( \pi \)-system is maximal. The bisected conformation is exemplified, for example, by the crystal structures of cyclopropylbenzene\(^{23,24}\) and of cyclopropyl acetophenone.\(^{25}\)

Bisected structures also occur in heteroaryl-substituted cyclopropanes such as 2-cyclopropylpyridine\(^ {26}\) and there now are two possibilities. In the case of heteroaryl cyclopropane \( 32 \), the two conformational possibilities are characterized by \( \tau = \angle(N1 - C2 - C_{\text{CP}} - H) = 0^\circ \) and \( \tau = 180^\circ \), and the crystal structure analysis shows that the first of these options is realized in the solid (\( \tau = 0^\circ \)).

Results of computational studies\(^ {27-31}\) show that the conformation of \( 32 \) observed in the solid state also is the preferred conformation in solution. As one of our potential collaborators, Dr. Glaser explored the potential energy surface of \( 32 \) with density functional theory, B3LYP/6-31+G(d), and also with second-order perturbation theory, MP2(full)/6-31+G(d), and the rotational energy profiles are shown in Figure 2.3.

![Relative Energy profile](image-url)
Figure 2.3: Rotational profiles of 32 computed as a function of the dihedral angle $\tau = \angle(N_O-C-C_{CP}-H)$ at the theoretical levels B3LYP/6-31+G(d) (blue) and MP2(full)/6-31+G(d) (green). Energies are given in kcal/mol relative to the $\tau = 180^\circ$ structure (M2).

The results of the potential energy analysis are consistent with the observed $^1$H-NMR spectrum of cyclopropyl hydrogen signals at 3.14 (m, 1H) and 1.36 (m, 4H) ppm.

![M1 and M2 structures](image)

Figure 2.4: B3LYP/6-31+G(d) optimized structures of conformers M1 and M2 of 32

The $^1$H NMR computational data support the preferred gas phase conformer also is preferred in solution. The chemical shifts and $J$ coupling values computed for M1 closely match the observed spectrum.

In conclusion, we found that the solid state conformation also is the preferred conformation in the gas phase and in solution. The crystallographic characterization of 1,2,4-benzotriazine di-N-oxide may be relevant to the biological property of 3-cyclopropyl-1,2,4-benzotriazine 1,4-dioxide radical where conformation might affect potential ring opening reactions. To the best of our knowledge, this is the first crystal structure determination of a 3-alkyl-1,2,4-benzotriazine di-oxide.
During the preclinical development of second generation analogues it has become clear that 3-alkyl-1,2,4-benzotriazine 1,4-di-N-oxides have activity comparable to TPZ.\textsuperscript{9,10} Hay et al. have shown that replacing the exo-cyclic amine with a non hydrogen donor such as an alkyl group can enhance the potency of the benzotriazinone 1,4 di-oxide class of antitumor agents. 3-methyl and 3-ethyl benzotrizine 1,4 dioxides display hypoxia selective cytotoxicities comparable to tirapazamine. These alkyl substituted N-oxide compounds may show better pharmacokinetics and extravascular transport properties (EVT) which are desirable conditions for an optimum drug candidate. Recently, Hay et al. have reported partition coefficients of alkyl substituted TPZ analogues with improved EVT and aqueous solubility. 3-methyl and 3-ethyl analogs showed higher logP values compared to TPZ.\textsuperscript{10} LogP is defined as the ratio of concentrations of a specific compound in an equilibrated mixture of aqueous and organic solvents such as water/octanol system. This factor is useful to determine how well the drug will be consumed and distributed in a human body. Consequently, we set out to determine the log P for 3-cyclopropyl-1,2,4-benzotriazine. The octanol water partition coefficient (logP) of 3-cyclopropyl 1,2,4-benzotriazine 1,4-dioxide was measured using the shake-flask method with analysis by UV-vis. TPZ and compound 32 (100 $\mu$M) were dissolved in 2 mL of 50 mM of sodium phosphate (pH 7) and 100 mM of NaCl solution. 2 mL of octanol was added, and vortexed thoroughly for about 4 min. Then it was centrifuged for 1 min. Two distinct aqueous and organic layers formed, and were subsequently collected and subjected to UV-vis spectroscopy. The concentrations of each compound in the partitioned layers
2.7 Redox-activated Hypoxia Selective DNA Cleavage by 3-Cyclopropyl 1,2,4-benzotriazine 1,4-dioxide (32)

From our understanding of TPZ chemistry and biology, we propose that the cytotoxicity of 32 might stem from its ability to damage DNA upon one electron reductive activation under hypoxic conditions. Thus, we first set out to examine the DNA cleaving ability and the hypoxia selectivity of 32 using supercoiled plasmid based DNA cleavage assays. NADPH:cytochrome P450 reductase or xanthine/xanthine oxidase enzyme systems were utilized for the one-electron reductive activation of 32, because these enzyme systems are extensively used for in-vivo and in-vitro studies respectively. In order to maintain the hypoxic conditions, molecular oxygen was removed from the stock solutions by three cycles of freeze-pump-thaw degassing, and the assay mixtures were prepared and incubated in an inert atmosphere glovebag. Reactions were incubated for 4-6 hours. Other additives such as catalase, superoxide dismutase, and desferal combination were used to inhibit possible molecular oxygen mediated DNA cleavage. We first set out to observe strand breaks in double-stranded, supercoiled plasmid DNA, mediated by compound 3. In this typical assay, reactive radicals convert supercoiled plasmid DNA (form I) to the open-circular form (form II) via a single strand cleavage; and the two forms are then separated by agarose gel electrophoresis. Abstraction of a hydrogen atom from the sugar backbone of DNA typically produces this type of strand cleavage. This DNA strand cleavage assay provided evidence that
compound 32 causes DNA strand cleavage when incubated with the NADPH:cytochrome P450 reductase enzyme system under hypoxic conditions. The yields of DNA strand breaks generated by 32 are comparable to those produced by TPZ (Figure 1A).

Figure 2.5: Cleavage of supercoiled plasmid DNA by 32 in the presence of NADPH:cytochrome P450 reductase as an activating system. All reactions contained DNA (1000 ng), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), and desferal (1 mM) and were incubated under anaerobic conditions at 24 °C for 16 h, followed by agarose gel electrophoretic analysis. Lane 1, DNA alone (S = 0.25 ± 0.02); lane 2, NADPH (500 µM) + reductase (1 mU/ml) (S = 0.33 ± 0.03); lane 3, TPZ (200 µM) only, (S = 0.32 ± 0.01); lanes 4, 32 (200 µM), (S = 0.32 ± 0.02); lane 5, TPZ (200 µM) + NADPH (500 µM) + reductase (S = 1.35 ± 0.10); lane 6, 32 (200 µM) + NADPH (500 µM) + reductase (S = 1.54 ± 0.16); lane 7, TPZ (250 µM) + NADPH (500 µM) + reductase (S = 1.79 ± 0.16); lane 8, 32 (250 µM) + NADPH (500 µM) + reductase (S = 1.96 ± 0.17); The average of at least three experiments. Figure 7b: The assays were done under similar reactions conditions and under aerobic conditions. There was no such significant cleavage above background. Lane 1, DNA alone (S = 0.42); lane 2, NADPH (500 µM) + reductase (1 mU/ml) (S = 0.42); lane 3, DNA + TPZ (250 µM) only, (S = 0.43); lane 4, DNA + 32 (250 µM), (S = 0.42); lane 5, DNA + TPZ (250 µM) + NADPH (500 µM) + reductase (S = 0.41); lane 6, DNA + 32 (250 µM) + NADPH (500 µM) + reductase (S = 0.43); The S-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation: \( S = - \ln f_1 \), where \( f_1 \) is the fraction of uncut, form I DNA remaining, where % form I = 100 – %form II, and the experiments were conducted in a manner such that only form I and II DNA were present.
CytP450 alone (no NADPH), or NADPH alone (no CytP450) did not produce significant DNA cleavage above background levels. Incubation with the enzymatic reducing system under aerobic conditions was unable to generate any DNA cleavage (Figure 1b). This result suggest that the amount of DNA cleavage generated by 32 was redox-activated and hypoxia selective.

DNA cleavage by 32 was significantly inhibited (Scheme) by classical radical-scavengers such as methanol, ethanol, t-butanol, DMSO, and mannitol (500 mM), (2.6). Overall, the data indicates that one-electron reduction of 32 under anoxic conditions leads to direct DNA strand cleavage via radical mechanisms, such as those characterized previously for TPZ.
**Figure 2.6:** Cleavage of supercoiled plasmid DNA by 32-TPZ in the presence of NADPH-cytochrome P450 reductase as an activating system. All reactions contained DNA (1000 ng, pGL-2 Basic), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), and desferal (1 mM) and were incubated under anaerobic conditions at 24 °C for 6 h, followed by agarose gel electrophoretic analysis. Lane 1, DNA alone (S = 0.36 ± 0.13); lane 2, NADPH (500 µM) + 32 (250 µM) (S = 0.33 ± 0.12); lane 3, 32 + NADPH (500 µM) + reductase (1mU/ml) (S = 2.1 ± 0.27); lanes 4-8, 32 (250 µM) + NADPH (500 µM) + reductase + methanol (500 mM, lane 4) (S = 0.50 ± 0.05); ethanol (500 mM, lane 5) (S = 0.58 ± 0.17); tert-butyl alcohol (500 mM, lane 6) (S = 0.77 ± 0.10); DMSO (500 mM, lane 7) (S = 0.71 ± 0.19); mannitol (500 mM, lane 8) (S = 0.58 ± 0.23); Lane 9, DNA + 32 (250 µM) alone (S = 0.35 ± 0.11); The average of at least three experiments. The S-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation: S = -ln f1, where f1 is the fraction of uncut, form I DNA remaining, where % form I = 100 – %form II, and the experiments were conducted in a manner such that only form I and II DNA were present.
2.8 Sequence Selectivity of DNA Cleavage by 3-Cyclopropyl 1,2,4-benzotriazine 1,4-di-oxide

To further characterization the DNA damage mediated by 32, we set out to determine the sequence-specificity of strand cleavage by 32. The sequence specificity of DNA strand cleavage by N-oxides compounds were typically examined a polyacrylamide gel electrophoresis assay.\textsuperscript{11,15,16} A 30-base pair, P\textsuperscript{32}-oligodeoxynucleotide duplex was treated with TPZ and 32 in the presence of the NADPH:cytP450R enzyme system under hypoxic conditions. The resulting P-labeled DNA fragments were resolved on a denaturing polyacrylamide sequencing gel and visualized by phosphorimager analysis.

\textbf{Figure 2.7:} Polyacrylamide gel analysis showing the sequence specificity of DNA cleavage by Tpz and 32 upon activation by NADPH/cytP450 reductase. Lane 1, DNA + TPZ + NADPH/cytP450 reductase, Lane 2, DNA + Cyp-TPZ + NADPH/cytP450 reductase; (B) Comparison of DNA-cleavage patterns generated by enzymatically activated TPZ, 3-cyclopropyl-TPZ, TPZ and 3-cyclopropyl-TPZ activated by NADPH:cytochrome P450 reductase enzyme system under anaerobic conditions. DNA cleavage reactions were performed on a 30 base pair 5\textsuperscript{'-}P-labeled oligodeoxynucleotide duplex (in the Experimental Section). Densitometer scans are from a portion of a 20% denaturing polyacrylamide gel and show the relative intensity of DNA cleavage at each base position. Lanes 1 and 2 were loaded with equal amounts (cpm) of labeled DNA and are plotted on the same vertical scale.
The sequencing gel analysis of DNA cleavage by compound 32 displayed a similar pattern of DNA cleavage at every base, which could resemble the typical fingerprint cleavage stemming from hydroxyl radical. We observed that DNA strand cleavage by reductively activated TPZ and 32 is comparable (Figure 2.7). DNA cleavage by 32 was also similar to an iron-EDTA-ascorbate system (data not shown) which presumably generates hydroxyl radical (Tullius). TPZ and 32 share a common reactive oxidizing species, such as the well known hydroxyl radical, for DNA strand cleavage under redox activated hypoxic conditions.

2.9 Redox Activated in vitro Metabolism of 32 under Hypoxic Conditions: 1-N-Oxide as the Major Metabolite

Our group and others have extensively studied on the metabolism of TPZ and various TPZ analogs.\textsuperscript{15,16,38,41} In presence of NADPH:cytochrome P450 reductase or xanthine/xanthine oxidase, TPZ yields the 3-amino-1,2,4-benzotriazine-1-oxide as the major metabolite. Identification of metabolites is an important part of the in vitro biochemical studies to understand the mechanism of DNA strand cleavage by TPZ, and by other various heterocyclic N-oxides.\textsuperscript{15,38} We examined the products generated by one-electron reductive activation of this compound by the enzyme NADPH:cytochrome P450 reductase under anaerobic conditions. The reaction mixture was extracted three times with ethyl acetate (200 µL each time), collected and air dried. Then sample was spotted on TLC plates (8 cm\textsuperscript{2} area). All the TLC plates were run at 40% ethyl acetate in hexanes. Then certain spot at R\textsubscript{f} = 0.8 with the silica gel was scraped off and redissolved in ethyl acetate and filtered through a regular column. It was dried and collected and compared
Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide

with the authentic standard sample. Thin layer chromatography (TLC) followed by mass spect., HPLC trace analysis reveals 1-N-oxide as a major product resulting from enzymatic activation of 32 (Figure 2.8).

Figure 2.8: HPLC trace of metabolism in the presence of 1-N-oxide produced in the reaction of metabolically activated 32

Scheme 2.4: Proposed mechanism of hypoxia-selective DNA cleavage by 3-cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide (32)
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**Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide**

The major metabolite displays the same retention time (around 5.5 min) as authentic standards of 3-cyclopropyl-1,2,4-benzotriazine 1-oxide (19). Thus, the major metabolite generated by one-electron bioreductive activation of 32 is common for heterocyclic N-oxides (Scheme 2.4). Large scale enzymatic reaction of xanthine/xanthine oxidase with compound 32 displayed the same metabolite as NADPH/CytP450 reductase systems.

2.10 Why Does Activated 3 Not Undergo Ring Opening Chemistry?

![Scheme 2.5: Proposed derailed mechanism of DNA damage by 32](image)

The extent of spin and charge delocalization on the radical intermediates, and the transition states for ring opening process should be considered first. It may be possible that, a greater amount of spin density is not transferred to cyclopropyl group which inhibits the ring opening. In spin density calculations (PUHF/6-31G*) of one electron
reduced TPZH radical, suggest that the unpaired electron is predominantly localized on the 1-N-oxide position. This data helps to explain why the cyclopropyl group of one electron reduced 32 does not undergo ring opening (Scheme 2.14). Ring opening may not be the fast because the spin density is localized in a nitroxyl type radical. Newcomb’s work supports the idea that a resonance-delocalized radical anion will not ring open if the spin is not located primarily at the carbonyl position adjacent to the cyclopropyl ring.

2.11 Conclusion

In conclusion, we found that the solid state conformation also is the preferred conformation in the gas phase and in solution. The crystallographic characterization of 1,2,4-benzotriazine di-N-oxide may be relevant to the biological property of 3-cyclopropyl-1,2,4-benzotriazine 1,4-dioxide radical where conformation might affect potential ring opening reactions. To the best of our knowledge, this is the first crystal structure determination of a 3-alkyl-1,2,4-benzotriazine di-oxide. We find that 3-cyclopropyl-1,2,4-benzotriazine 1,4-di-N-oxide behaves like TPZ. Interestingly, the cyclopropyl ring opening does not occur, at least in part, may be due to the unpaired electron residing predominantly on nitroxy moiety of bioreductively reduced 3 radical, which adds useful new information to our understanding of cyclopropyl ring opening reactions. Two mechanisms have been proposed to explain TPZ-mediated DNA damage. We have evidence that the DNA damage by TPZ involves homolytic fragmentation of the N-OH bond in the neutral drug radical. In order to facilitate the homolytic N-OH bond cleavage, one can assume that the spin density at C3 position TPZ should be enough to initiate this homolysis of the N-OH bond in TPZH radical. From our current studies, we find that well known cyclopropyl ring opening does not occur in activated X, simply because the spin is not
enough to cause the ring opening. The Homolytic fragmentation and no ring-opening for 32, spin density only can not answer the facts. We need to consider the thermodynamic contribution, such as energy of activation. N-OH fragmentation is quantified and considered to be thermodynamically favorable.\textsuperscript{35} It, is possible that the ring opening reaction in activated 32 is not thermodynamically feasible, which derails the chemistry toward homolytic fragmentation.

2.12 Experimental Section

Materials were of the highest purity available and were obtained from following sources: sodium phosphate, mannitol, xanthine, DMSO, and TLC plates from Aldrich
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Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide

Chemical Co. (Milwaukee, WI); NADPH, desferal, cytochrome P450 reductase, catalase, calf thymus DNA, and superoxide dismutase (SOD) from Sigma Chemical Co. (St. Louis, MO); xylene cyanol, bromophenol blue, formamide, and urea from United States Biochemical; T4 polynucleotide kinase from New England Biolabs; [γ-32 P]-dATP from Perkin-Elmer Life Sciences; oligonucleotides were purchased from Integrated DNA Technologies; acrylamide and bisacrylamide from Roche Diagnostics; xanthine oxidase from Roche Diagnostics; agarose from Seakem; HPLC grade solvents (acetonitrile, methanol, ethanol, tert-butyl alcohol, ethyl acetate, hexane, and acetic acid) from Fischer (Pittsburgh, PA); ethidium bromide from Roche Molecular Biochemicals (Indianapolis, IN); Silica gel (0.04- 0.063 mm pore size) for column chromatography from Merck. The plasmid pGL2BASIC was prepared using standard protocols. Tirapazamine (1, TPZ), 4, and 32 were synthesized according to literature methods. High resolution mass spectroscopy was performed at the University of Illinois Urbana Champaign Mass Spectroscopy facility and low resolution mass spectroscopy were performed at the University of Missouri-Columbia.

2.13 Oxidation of 3-cyclopropyl-1,2,4-benzotriazine (2) with m-Chloroperbenzoic Acid.

3-Cyclopropyl-1,2,4-benzotriazine 19 (50 mg, 0.25 mmol) was dissolved in 10 mL of dichloromethane at room temperature. To this solution, meta-chloroperbenzoic acid (mCPBA, 2-6 equivalent) was added and the resulting reaction mixture stirred at room temperature until all starting material was consumed. The solvent was then evaporated and the residue was purified using gravity column chromatography on silica.
gel eluted with ethyl acetate/hexanes (1:1) to provide 32 as deep yellow powder. $^1$H-NMR (CDCl$_3$, 500 MHz): $\delta$ 8.55 (dd, $J = 8.5$, 1 Hz, 1H), 8.43 (dd, $J = 8.5$, 1 Hz, 1H), 8.00 (ddd, $J = 8.5$, 7, 1 Hz, 1H), 7.79 (ddd, $J = 8.5$, 7, 1 Hz, 1H), 3.14 (tt, $J = 8.0$, 5.0 Hz, 1H), 1.36 (m, 4H); $^{13}$C-NMR (125 MHz, CDCl$_3$): $\delta$ 157.1, 139.0, 135.4, 133.9, 131.1, 121.5, 119.4, 10.1, 9.3; HRMS (ESI) $m/z$ calc for C$_{10}$H$_{10}$N$_3$O$_2$ (MH$^+$) 204.0773, found 204.0765.

![Scheme 2.6](image)

**Scheme 2.6:** Synthetic route for the preparation of 3-cyclopropyl-1,2,4-benzotriazine 1,4-di-N-oxide

### 2.14 Crystallography

Slow evaporation of dilute solutions of 3 in ethyl acetate-hexane afforded crystals suitable for X-ray diffraction analysis. Data was collected on Bruker SMART system at 173 K. Crystal structures were solved by using SHELX programs.$^{12,13}$ Details of the data collection and of the structure refinement are provided in Table 2.2.
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Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide

Table 2.1: Crystallographic data

<table>
<thead>
<tr>
<th>Compound 32</th>
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<tbody>
<tr>
<td>Chemical formula</td>
</tr>
<tr>
<td>CCDC no.</td>
</tr>
<tr>
<td>Color/shape</td>
</tr>
<tr>
<td>Formula weight</td>
</tr>
<tr>
<td>Crystal system</td>
</tr>
<tr>
<td>Space group</td>
</tr>
<tr>
<td>Temperature, K</td>
</tr>
</tbody>
</table>
| Unit cell dimensions | \[a = 16.6306(12) \text{ Å} \]
\[b = 7.799(5) \text{ Å} \]
\[c = 16.0133(11) \text{ Å} \]
\[\alpha = 90^\circ \]
\[\beta = 119.0440(10)^\circ \]
\[\gamma = 90^\circ \]
| Volume, Å³ | 1815.8(2) |
| Z | 8 |
| Density (calculated), mg/m³ | 1.494 |
| Absorption coefficient, mm⁻¹ | |
| Diffractometer/scan | Bruker SMART/CCD area detector |
| θ range for data collection, deg. | 2.8 to 27.13 deg. |
| Reflections measured | |
| Independent/observed reflections | |
| Data/restraints/parameters | 2005/0/140 |
| Absorption correction | Semi-empirical |
| Goodness of fit on F² | 1.066 |
| \(T_{\text{min}}, T_{\text{max}}\) | 0.73, 0.98 |
| Final R indices \([I > 2\sigma (I)]\) | R1 = 0.0581, \(\omega\)R² = 0.1495 |
| R indices (all data) | R1 = 0.0721, \(\omega\)R² = 0.1614 |
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**Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide**

**Table 2.2:** Final Coordinates and Equivalent Isotropic Displacement Parameters of the non-Hydrogen atoms for compound 32

<table>
<thead>
<tr>
<th>Atom</th>
<th>x</th>
<th>Y</th>
<th>z</th>
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<td>1959</td>
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<td>12598</td>
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Table 2.3: Bond Distances (Å) for compounds 32

<table>
<thead>
<tr>
<th>Bond</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
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<td>O(1)−N(2)</td>
<td>1.270(2)</td>
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Table 2.4: Bond Angles (deg) for compounds 32

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### Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide

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### Table 2.5: Anisotropic displacement parameters (Å² x 10³) for 3.
The anisotropic displacement factor exponent takes the form: \(-2 \pi^2 \sum U_{ij} a_i a_j \) where \( a_i a_j \) is the \( i \)-th and \( j \)-th components of the unit cell.

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Chapter 2

**Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide**

**Table 2.6:** Hydrogen coordinates (\(x \times 10^4\)) and isotropic displacement parameters (\(A^2 \times 10^3\)) for 32

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**Table 2.7:** Torsion angles [deg] for 32

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Symmetry transformations used to generate equivalent atoms
2.15 Partition Coefficients:

The octanol water partition coefficient (P) of 3-cyclopropyl 1,2,4-benzotriazine 1,4-dioxide was measured using the shake-flask method with analysis by UV-vis. The octanol-water partition coefficients were measured by using mechanical shake flask method. Tpz and compound 3 were dissolved in 2 mL of 50 mM of NaP (pH 7) and 100 mM of NaCl solution. 2 mL of octanol was added and vortexed well for about 4 min. Then it was centrifuged for 1 min. Two distinct layers were formed. Aqueous and organic layers were collected and subjected to UV-vis spectroscopy. Concentration of each compound in each layer was calculated. We obtained -0.27 for TPZ and logP= 0.26 for cyp-tpz.

2.16 Cleavage of Supercoiled Plasmid DNA.

In a DNA cleavage assay, a solution of 30 µL containing supercoiled plasmid DNA (1000 ng), NADPH (500 µM), cytochrome P450 reductase (1 mU), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM) were incubated with di-N-oxide (32-TPZ or TPZ, 250 µM). In all DNA damage reactions, individual components, except DNA, NADPH, and enzymes, were deoxygenated by using three cycles of freeze-pump-thaw in pyrex tubes and then torch-sealed under high vacuum. Sealed tubes were scored, and opened in a glove bag filled with argon, and used to prepare individual reactions. Enzymes, NADPH, and DNA were diluted with degassed water in the glove bag to prepare stock solutions. Reactions were initiated by adding cytochrome P450 reductase, then wrapped with aluminium foil to prevent exposure to light, and incubated for 3 h in the glove bag at room temperature
Following incubation, the reactions were stopped by adding 5 µL of 50% glycerol loading buffer, and the resulting mixture was loaded onto a 0.9% agarose gel. The gel was electrophoresed for approximately 2.5 h at 82 V in 1 X TAE buffer then stained in a solution of aqueous ethidium bromide (0.3 µg/mL) for 3 h. DNA in gel was visualized by UV-transillumination, and the amount of DNA in each band was quantified using an Alpha Innotech IS-1000 digital imaging system. DNA-cleavage assays containing radical scavengers were performed as described above with the exception that radical scavengers such as a methanol, ethanol, tert-butyl alcohol, DMSO, or mannitol (500 mM) were added to the reaction before addition of cytochrome P450 reductase. To prevent the background DNA damage from superoxide radical, superoxide dismutase, catalase and desferal were added to reactions. Concentration dependent DNA cleavage assays for TPZ, and 32 were also performed similar manner and incubated for 4 h.
Bioreductively Activated DNA Damage Studies of 3-Cyclopropyl-1,2,4 benzotriazine 1,4-di-oxide

**Figure 2.9:** Cleavage of supercoiled plasmid DNA by TPZ in the presence of NADPH:cytochrome P450 reductase as an activating system. All reactions contained DNA (1000 ng), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), and desferal (1 mM) and were incubated under anaerobic conditions at 24 °C for 6 h, followed by agarose gel electrophoretic analysis. Lane 1, DNA alone (S = 0.28 ± 0.12); lane 2, NADPH (500 µM) + TPZ (250 µM) (S = 0.32 ± 0.11); lane 3, TPZ + NADPH (500 µM) + reductase (1mU/ml) (S = 2.01 ± 0.42); lanes 4-8, TPZ(250 µM) + NADPH (500 µM) + reductase + methanol (500 mM, lane 4) (S = 0.37 ± 0.09); ethanol (500 mM, lane 5) (S = 0.36 ± 0.10); tert-butyl alcohol (500 mM, lane 6) (S = 0.47 ± 0.14); DMSO (500 mM, lane 7) (S = 0.49 ± 0.06); mannitol (500 mM, lane 8) (S = 0.39 ± 0.06); The average of at least three experiments. The S-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation: S = -ln f_i, where f_i is the fraction of uncut, form I DNA remaining, where % form I = 100 – %form II, and the experiments were conducted in a manner such that only form I and II DNA were present.

**Figure 2.10:** Cleavage of supercoiled plasmid DNA by TPZ and 32 in the presence of various amount of NADPH:cytochrome P450 reductase as an activating system. All reactions contained DNA (1000 ng, pGL-2 Basic), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 µg/mL), superoxide
dismutase (10 µg/mL), and desferal (1 mM) and were incubated under anaerobic conditions at 25 °C for 5 h, followed by agarose gel electrophoretic analysis. Lane 1, DNA alone (S = 0.39); lane 2, DNA + NADPH (500 µM) + reductase (1mU/ml) (S = 0.40); lane 3, DNA + TPZ (200 µM) (S = 0.42); lane 4, DNA + CypTPZ (200 µM) (S = 0.41); lane 5, DNA + NADPH (500 µM) + 1/4 x reductase+ TPZ (200 µM) (S = 0.77); lane 6, DNA + NADPH (500 µM) + 1/2 x reductase+ TPZ (200 µM) (S = 0.64); lane 7, DNA + NADPH (500 µM) + 3/4 x reductase+ TPZ (200 µM) (S = 0.76); lane 8, DNA + NADPH (500 µM) + 1 x reductase+ TPZ (200 µM) (S = 1.28); lane 9, DNA + NADPH (500 µM) + 1.25 x reductase+ TPZ (200 µM) (S = 1.9); lane 10, DNA + NADPH (500 µM) + 1/4 x reductase+ 32(200 µM) (S = 0.56); lane 11, DNA + NADPH (500 µM) + 1/2 x reductase+ 32(200 µM) (S = 0.61); lane 12, DNA + NADPH (500 µM) + 3/4 x reductase+ 32 (200 µM) (S = 1.02); lane 13, DNA + NADPH (500 µM) + 1 x reductase+ 32 (200 µM) (S = 1.43); lane 14, DNA + NADPH (500 µM) + 1.25 x reductase+ CypTPZ (200 µM) (S = 1.82); The average of two experiments. The S-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation: \[ S = -\ln f_i \], where \( f_i \) is the fraction of uncut, form I DNA remaining, where % form I = 100 – %form II, and the experiments were conducted in a manner such that only form I and II DNA were present.

2.17 Metabolite Identification by TLC, MS and HPLC Method

In a metabolic studies assay, a solution of cyp-TPZ or (500 µM) and desferal (1 mM) in sodium phosphate buffer of pH 7 was deoxygenated by three freeze-pump-thaw cycles and then torch-sealed under vacuum. The sealed tube was scored before being transferred to an argon filled glove bag. The tube was then opened and, NADPH (1 mM), and cytochrome P450 reductase (10 mU), were added and samples were incubated in an argon filled glove bag at 25 °C for 4 h. The proteins were then removed by centrifugation through Amicon Microcon (YM3) filters. The identity of the metabolites was first confirmed by thin layer chromatography using 40:60 EtOAc/Hexanes followed by mass spectrometry (MS) analysis. The reaction mixture was spotted on TLC and then the major
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metabolite compared with authentic standard 3-cyclopropyl-benzotriazine-1-N-oxide. The major spot on the TLC was scraped and dissolved with EtOAc. Then the organic silica mixture was put on a clean column in order to get rid of silica gel. Then the organic solvent was evaporated and sample was given to mass spect. which ultimately showed that intact 3-cyclopropyl-benzotriazine 1-N-oxide is the major product. Also, the filtrate was analyzed by normal phase HPLC employing a amino Rainin Microsorb-MV column (5 µm particle size, 100 Å pore size, 25 cm length, 4.6 mm i.d.) eluted with a isocratic mobile phase hexane/2-propanol (100 : 3), at a flow rate of 1mL/min, and the products were monitored by UV-absorbance at 270 nm. The major product was identified as 3-cyclopropyl benzotriazine-1-N-oxide by co-injection of reaction mixture with authentic compounds.

### 2.18 Confirmation of Metabolism by UV-vis

This method is also very useful to check how whether metabolism occurred and even one can identify the amount of metabolism by using handy and fast method. Typically, HPLC and LC-MS methods are used to indentify metabolites generated from drugs. Combination of TLC, UV-vis and MS can be faster, and cheaper method for the identification of drug metabolites. In a metabolic studies assay, a solution of TPZ and 32 or (500 µM) and desferal (1 mM) in sodium phosphate buffer of pH 7 was deoxygenated by three freeze-pump-thaw cycles and then torch-sealed under vacuum. The sealed tube was scored before being transferred to an argon filled glove bag. The tube was then opened and, NADPH (1 mM), and cytochrome P450 reductase (10 mU), were added and samples were incubated in an argon filled glove bag at 25 ºC for 4 h. The proteins were then removed by centrifugation through Amicon Microcon (YM3) filters. The reaction
mixture was extracted with ethyl acetate and checked with UV-vis, and compared with 32. As a good control, TPZ was subjected to metabolism side by side and checked in UV-vis. Consumption of TPZ and 32 was observed, and new absorption spectra were observed at 405 and 360 nm, which are typically spectra of mono-N-oxide of TPZ and 32.

**Figure 2.11:** UV-vis spectra of enzymatic reactions of 32 and TPZ
2.19 Sequence Specificity Experiments

A 30-base pair oligonucleotide with a sequence of 5′-GTC ACGTGCTGACGACGCTGCTGAGCCT-3′ was 5′-end labeled with $^{32}$P using [$\gamma$-$^{32}$P] dATP and T4 polynucleotide kinase and was purified on a 20% denaturing polyacrylamide gel. The labeled single strand oligonucleotide was then annealed with its complimentary strand by heating the mixture to 90 °C followed by slow cooling to room temperature for overnight. For this sequence specificity experiment, oligonucleotide, NADPH, cytochrome P450 reductase and TPZ, $^{32}$ were placed inside a dialysis bag and suspended in a sodium phosphate buffer solution containing the enzyme substrate and TPZ, $^{32}$. In this way the steady concentration of NADPH and TPZ, $^{32}$ can be maintained throughout the reaction time and the amount of cleavage is moderately increased. Cleavage reactions by TPZ, $^{32}$ were run in deoxygenated aqueous buffer (three freeze-pump-thaw cycles) inside an inert atmosphere glove bag. Degassed reaction mixture containing the duplex oligonucleotide (200,000 cpm), TPZ, $^{32}$ (250 µM), sodium phosphate buffer (10 mM, pH 7.0), desferal (1 mM), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), NADPH (1 mM) and cytochrome P450 reductase (5 mU) in a total volume of 100 µL was pipetted into a Slide-A-Lyzer minimal unit (Pierce, 3000 MW cutoff). The dialysis unit was then placed with a floater into 2 mL of solution containing TPZ, $^{32}$ (250 µM), NADPH (1 mM), sodium phosphate buffer (10 mM, pH 7.0), and desferal (1 mM) and gently stirred for 16 h in a glove bag filled with argon, followed by the removal of DNA from the dialysis unit. The oligonucleotide was
precipitated using 0.3 M sodium acetate, 70% ethanol (final concentration) and 5 µg of carrier DNA (herring sperm), and then washed three times with 80% ethanol/water. The dried oligonucleotide fragments were dissolved in formamide loading buffer, heated at 90 ºC for 5 min, then immersed in ice water and equal number of counts were loaded in each lane of 20% denaturing polyacrylamide gel. The gel was electrophoresed for 3 h at 1200 V in 10×TBE buffer. The DNA fragments in the gel were visualized by phosphorimager analysis (Molecular Imager®FX, Imaging Screen-K, cat 170-7841, Bio-Rad, using Quantity One® Version 4.2.3, Bio-Rad). The Maxam-Gilbert A + G and G-reactions were performed following standard protocols. DNA cleavage by iron EDTA/H$_2$O$_2$/ascorbate was carried out as described by Tullius and co-workers.
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$^1$H NMR Spectra of 3-Cyclopropyl-benzotriazine 1,4-dioxide (32)
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$^{13}$C NMR Spectra of 3-Cyclopropyl-benzotriazine 1,4-dioxide (32)
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13C depth 135, CH and CH3 up, CH2 down

JU-cyptx-dioxide-expt2-in ccl3
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Molecular Mechanism of DNA Damage by 3-Methyl-1,2,4-benzotriazine 1,4-di-N-oxide

3.1 Introduction

The Benzotriazine 1,4–dioxide class of compounds are under active clinical trials as redox-activated, hypoxia selective cytotoxic agents. 3-Amino 1,2,4 benzotriazine 1,4-dioixde (TPZ) is the lead compound and has been in clinical trials.\textsuperscript{1,2} The compound was first characterized as a cytotoxic agent 1985 by Martin Brown and his coworkers. For the past two decades, understanding its mechanism of action is still under invetigation because of its complex, radical-mediated cytotoxicity under very low oxygen concentration in tumor cells.\textsuperscript{3-6} For an example, understanding the mechanism of antitumor drug bleomycin is still under active investigation over two decades.\textsuperscript{7} In presence of intracellular reductive enzyme TPZ typically gets reduced by one electron to
yield radical anion. The radical anion gets protonated, and is in equilibrium with its radical anion form. Two possible mechanisms are well accepted for the “toxic” chemistry of bio-reductively activated TPZ which occurs under hypoxic conditions. Brown and coworkers initially suggested the protonated form of the TPZ neutral radical 4b is responsible for DNA damage.\textsuperscript{8,9,10,11}

For over a decade our group has done extensive biochemical and mechanistic studies on TPZ and have evidence that activated TPZ undergoes homolytic fragmentation, and releases the classical DNA damaging species hydroxyl radical.\textsuperscript{3,4,12-14}

\begin{center}
\textbf{Scheme 3.1:} Proposed mechanism of DNA damage by TPZ radical
\end{center}

Our group has provided the first structural characterization of nucleobase damage mediated by TPZ. Our group found nucleobase hydroxylation which is typically signature product for the reaction of a hydroxyl radical with DNA bases.\textsuperscript{13} In addition, nonspecific abstraction of hydrogen atom from the deoxyribose backbone of duplex DNA by metabolically activated TPZ, displays end products which mirror the products produced by an oxidative DNA damage of hydroxyl radical under aerobic conditions (Scheme 3.2).\textsuperscript{14} We have shown that enzymatically activated TPZ generates DNA sugar radicals
which degrade in presence of TPZ. This observation parallels oxidative DNA damage by hydroxyl radical in presence of oxygen. Thus, TPZ itself, mimics oxygen under hypoxic conditions.\textsuperscript{15}

\textbf{Scheme 3.2:} Hydroxyl radical mediated signature products stemming from DNA sugar damage

EPR experiments, using DMPO as a trapping agent, computational experiments, and density functional theory, strongly support the idea that the protonated form of activated TPZ undergoes N-O fragmentation to release a hydroxyl radical.\textsuperscript{16,17}

Recently, Denny and coworkers, suggested an alternative mechanism which almost parallels oxidative DNA damage chemistry mediated by hydroxyl radical. In their proposed scheme the protonated TPZ neutral radical (4b) upon dehydration yields benzotriazinyl radical (36a or 36b), and ultimately responsible for DNA strand cleavage.\textsuperscript{5,6} Denny and coworkers have generated 36a or 36b from 3-amino 1,2,4-benzotriazine 1-N-oxide in order to prove their proposed dehydration mechanism. In

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recent year, Shinde et al. have shown the evidence of generation of nitrogen centered and carbon centered radicals by spin trapping method under redox-activated hypoxic conditions, which might be responsible for DNA stand scission.\textsuperscript{6} However, the exact nature of reactive species that cause in vivo double strand DNA damage is a matter of debate for TPZ. It is very reasonable to speculate that it may be the combinations of two or more radical species which might be operative for cellular DNA damage in a complex biological matrix. So, unraveling the exact nature of radical species responsible for DNA damage under hypoxia, is still an active investigation.

Scheme 3.3: Two possible pathways of generating reactive radical intermediates from TPZH radical
3.11 Reexamination of the Molecular Mechanism of TPZ Action

We utilized 3-methyl-1,2,4-benzotriazine-di-N-oxide (12) as a mechanistic tool to reinvestigate the degradation of TPZ action under hypoxia. We believe in the hydroxyl radical fragmentation based on our evidence, but we wanted to consider Denny’s suggested mechanism too. Thus, we set out to test the plausible existence of benzotriazinyl radical, which might be responsible for DNA damage.

3.12 Redox-activated, Hypoxia Selective DNA Cleavage by 3-Methyl 1,2,4-benzotriazine 1,4-dioixde (12)

From our understanding of tirapazamine and other-N-oxide compounds, we speculated the cytotoxicities of 3-methyl 1,2,4-benzotriazine 1,4 dioixde (12) might stem from its ability to damage DNA upon one electron reductive activation under hypoxic conditions. Thus, we first set out to examine the double stranded DNA cleaving ability, and the hypoxia selectivity of 12 using supercoiled plasmid based DNA cleavage assays. NADPH:cytochrome P450 reductase or xanthine/xanthine oxidase (X/XO) enzyme systems were utilized for the one-electron reductive activation of 12, as these enzyme systems are extensively used for in vivo and in vitro studies respectively.\(^{18-21}\) In order to establish the hypoxic conditions, molecular oxygen was removed from stock solutions by three cycles of freeze-pump-thaw degassing and the assay mixtures were prepared and incubated in an inert atmosphere glovebag. Reactions were incubated for 4-6 hours, and other additives such as catalase, superoxide dismutase, and desferal combinations were used to inhibit molecular oxygen mediated DNA cleavage if any exists. In a typical assay, a reactive radical converts supercoiled plasmid DNA (form I) to the open-circular form.
(form II) via a single strand cleavage, and are then separated using agarose gel electrophoresis.\textsuperscript{22-24} Abstraction of a hydrogen atom from the sugar backbone of DNA typically produces this type of strand cleavage.\textsuperscript{25-27} The DNA strand cleavage assay showed evidence that compound \textbf{12} causes DNA strand cleavage when incubated with the NADPH:cytochrome P450 reductase enzyme system under hypoxic conditions (Figure ). The yields of DNA strand breaks generated by \textbf{12} were relatively low compared to those produced by TPZ in the presence of the X/XO system. The X/XO can carry out two electron reduction and perhaps \textbf{12} is probably more susceptible to two electron reduction which can cause low levels of strand cleavage (Fig. 3.1).

![Figure 3.1](image)

\textbf{Figure 3.1}: Cleavage of supercoiled plasmid DNA by various concentration of TPZ and \textbf{12} in presence of X/XO activating system. Supercoiled plasmid DNA (1000 ng) incubated with TPZ and Me-TPZ (50-250 \textmu M), xanthine (500 \textmu M), xanthine oxidase (0.4 U/ml), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 \textmu g/ mL), superoxide dismutase (10 \textmu g/mL), and desferal (1 mM) under anaerobic conditions at 25 \textdegree C for 6 h, followed by agarose gel electrophoretic analysis. The average of two experiments. The S-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation: \( S = -\ln f_i \), where \( f_i \) is the fraction of uncut, form I DNA remaining, where \% form I = 100 – \%form II, and the experiments were conducted in a manner such that only form I and II DNA were present.
However, the yields of DNA strand breaks generated by 12 are comparable to those produced by TPZ with the NADPH/CytP450R enzymatic systems (Fig. 3.2 and 3.2a). With the cytochrome P450 reductase alone, and NADPH alone, did not produce significant DNA cleavage above background. Incubation with the enzymatic reducing system under aerobic conditions was unable to produce DNA cleavage (data not shown), which implied that the amount of DNA cleavage generated by 12, was redox-activated and hypoxia selective.

**Figure 3.2:** Comparison of DNA cleavage efficiency of TPZ and Me-TPZ

**Figure 3:** Cleavage of supercoiled plasmid DNA by various concentration of TPZ and 12 in presence of NADPH/cytochrome P450R activating system. Supercoiled plasmid DNA (1000 ng) incubated with TPZ and Me-TPZ (50-250 µM), NADPH (500 µM), reductase (1 U/ml), sodium phosphate buffer (50 mM, pH
DNA cleavage by 12 was significantly inhibited by classical radical-scavengers such as methanol, ethanol, t-butanol, DMSO, and mannitol (500 mM). Overall, the data indicate that one-electron reduction of 12 under anoxic conditions leads to direct DNA strand cleavage via radical mechanisms such as those characterized previously for TPZ.

**Figure. 3.4:** Mechanism 12 in the presence of NADPH:cytochrome P450 reductase as an activating system. All reactions contained DNA (1000 ng, pGL-2 Basic), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 µg/ mL), superoxide dismutase (10 µg/mL), and desferal (1 mM) and were incubated under anaerobic conditions at 25 °C for 6 h, followed by agarose gel electrophoretic analysis. Lane 1, DNA alone; lane 2, Me-TPZ (250 µM) + NADPH (1mM) + reductase (1mU/mL); Lane 3, MeTPZ (250 µM) + NADPH only; Lane 4, 12 (250 µM) + reductase only; Lane 5, 12 (250 µM) + NADPH + reductase; Lanes 6-10, 12 (250 uM) + NADPH + reductase + methanol (100 mM); ethanol (100 mM); t-butanol (100 mM); DMSO (100mM); mannitol (100mM); Lane 11, 12 (250 µM) only. The average of at least three experiments. The S-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation: $S = -\ln f_1$, where $f_1$ is the fraction of uncut, form I DNA remaining, where % form I = 100 – %form II, and the experiments were conducted in a manner such that only form I and II DNA were present.
3.13 Sequence Specificity of DNA Cleavage by 4 and 12

We hypothesize that, if benzotriazine di-oxides release a hydroxyl radical, the DNA cleavage of these compounds should be sequence independent.²⁸ To examine our hypothesis, we also examined the sequence-specificity of DNA strand cleavage by 12. In these experiments, a 30 base pair, 5'-³²P-labeled oligodeoxynucleotide duplex, was treated with 12 and the NADPH:cytochrome P450 reductase enzyme system under anoxic conditions. Following the DNA-damage reaction, the resulting ³²P-labeled DNA fragments were resolved on a denaturing polyacrylamide sequencing gel and visualized by phosphorimager analysis. The relevant lanes from the gel are displayed as densitometry traces, in which each cleavage band appears as a peak (Figure 3.4). We find that DNA strand cleavage by reductively activated 12, occurs at every base pair in the duplex (Figure 3.4B). The pattern of sequence-independent DNA strand cleavage caused by 12 closely resembles that generated by TPZ (Figure 3.4C), and is generally characteristic of a highly oxidizing, small, diffusible species such as hydroxyl radical.²⁸ We also compared the DNA strand cleavage of 12 and TPZ to that of an iron-EDTA system which generates hydroxyl radical (or a functionally equivalent species, Figure 3.4D).²⁸ Comparison with the iron-EDTA cleavage lane reveals both TPZ and 12 display
Figure 3.5: (A–D) Comparison of DNA-cleavage patterns generated by enzymatically activated TPZ, 12, and iron-EDTA. (A) Control; NADPH: cytochrome P450 reductase enzyme system, (B) TPZ activated by NADPH:cytochrome P450 reductase enzyme system under anaerobic conditions, (C) compound 12 activated by NADPH:cytochrome P450 reductase enzyme system under anaerobic conditions and, (D) the hydroxyl radical-generating Fe-EDTA system under aerobic conditions. DNA cleavage reactions were performed on a 30 base pair 5′-32 P-labeled oligodeoxy nucleotide duplex as described in the Experimental Section. Densitometer scans are from a portion of a 20% denaturing polyacrylamide gel and show the relative intensity of DNA cleavage at each base position. Lanes A, B and C were loaded with equal amounts (cpm) of labeled DNA and are plotted on the same vertical scale. Lane D, provided for comparison, is not plotted on the same vertical scale as A, B, and C. (E–H) Comparison of DNA-cleavage patterns generated by enzymatically activated TPZ and hydroxyl radical generated by the anaerobic photolysis of H₂O₂ in sodium phosphate buffer containing 40 mM NaCl. (E) Control, DNA treated with the NADPH:cytochrome P450 reductase enzyme system under anaerobic conditions in the absence of TPZ (F)
Control, DNA subjected to photolysis under anaerobic conditions, (G) TPZ activated by NADPH:cytochrome P450 reductase enzyme system under anaerobic conditions in the same buffer and salt conditions employed for the peroxide photolysis, (H) DNA strand cleavage by hydroxyl radical generated via photolysis of H₂O₂. Lanes E and G and F and H were loaded with equal amounts (cpm) of labeled DNA and are plotted on the same vertical scale so they can be compared.

some preference for cleavage at purine residues in the duplex. This result raises the possibility that iron-EDTA is not an appropriate control because the system operates under aerobic conditions. Thus, we were driven to examine the sequence specificity of DNA strand cleavage by hydroxyl radical. For this purpose, we employed a method developed by MacGregor involving the generation of hydroxyl radical via photolysis of hydrogen peroxide. This method is attractive because it can be carried out effectively under anaerobic conditions and there is no possibility for complications arising from any organic fragment that serves as a “delivery vehicle” for the HO• cleaving agent. In this experiment, we find that the anaerobic photolysis of H₂O₂ yields strand cleavage with no base specificity (Figure 3.4H). Indeed the result closely resembles DNA strand cleavage by the iron-EDTA system (compare Figure 3.4D and 3.4H). We carried out the photolysis reactions under the salt conditions (40 mM NaCl) employed by MacGregor. Therefore, it was necessary to examine strand cleavage by TPZ under identical buffer and salt conditions for comparison (Fig. 3.4G). Control reactions under these conditions show little cleavage by photolysis in the absence of hydrogen peroxide (Fig. 3.4F) or the enzyme system in the absence of TPZ (Fig. 3.4E). It is clear that the N-oxide class of compounds cause DNA strand cleavage at every nucleotide, but with a little preference for cleavage at purine residues, under our reaction conditions. We speculate that
bioreductively activated benzotriazine $N$-oxides get metabolized with release of a hydroxyl radical. If that is the case, why is the DNA sequence specificity by the $N$-oxides not a mirror image of anaerobic hydroxyl radical? According to our experimental data, generation of the benzotriazinyl radical could be a minor process. If so, small concentration of benzotrizinyl radical could contribute to sequence specificity under our reaction conditions.\textsuperscript{29a,30} Benzotrizine has been shown to have redox potential appropriate for oxidation of purine bases in nucleosides.\textsuperscript{30a}

Alternatively, an intermediate hydroxyl radical adduct with deoxypurine may undergo complex secondary reactions with di-$N$-oxides, and other metabolites, which ultimately may lead to the generation of an extra strand cleavage in our experiments.

\textbf{Scheme 3.4} Generation base-labile DNA lesion form hydroxyl radical mediated DNA damage
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Typically, addition of hydroxyl radical to the 5,6-double bond of guanine, followed by loss of water, yields (G-H)• radical (Scheme 3.4). Further oxidation of (G-H)• by superoxide radical or other oxidizing agent is thought to generate hydantoin or imidazolone products that may cause DNA strand cleavage. It is also known that oxidation-induced sugar damage affords sites leading to frank strand scission or heat-labile sites (Scheme 3.5).

![Scheme 3.5: Possible selective oxidation at 8-oxoG lesions](image)

3.14 Compound 12 yields 1-N-oxide as a Major Metabolite

Incubation of 12 with NADPH:cytochrome P450 reductase under anaerobic conditions yields multiples metabolites. Metabolites were characterized by HPLC, and
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LC/MS. HPLC traces showed that 12, upon bioreductive activation under hypoxia yields
the 1-N-oxide as a major metabolite and no-N-oxide as a minor metabolite analogous to
TPZ (Figures 3.6). Having found this, we speculate TPZ and 12 must share a common
mechanism of action of DNA strand cleavage.

Scheme 3.6: In vitro metabolism of benzotriazine di-N-oxides yield two metabolites

3.15 Isotopic Labeling Studies of 12 Support N-OH Homolysis Mechanism

To investigate the existence of dehydration mechanism, we did isotopic labeling
studies. We proposed that bioreductive activation studies in presence of a deuterated
medium would yield metabolite which could be different in mass. If dehydration occurs
in activated 12,

Scheme 3.7: Examination of the existence of dehydration chemistry in 12 in D₂O/MeOD
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The benzotriazinyl radical generated from the radical 41, should be primarily trapped by deuterated solvent (Scheme 3.7). On the other hand, if 12 primarily releases hydroxyl radicals, ideally no deuterium incorporation should be observed. By LC/MS experiments, Junnotula and his colleagues showed that only around 5% deuterium incorporation occurs in the major metabolite of 12, which strongly support the release of hydroxyl radical mechanism.

Scheme 3.8: Proposed mechanism of degradation of 12 under bioreductive activation

3.16 Conclusion

In this chapter, we presented evidence which support the hypothesis that upon one-electron reductive-activation, 1,2,4-benzotriazine-1,4-dioxides primarily results in the releases of hydroxyl radicals. However, the exact nature of reactive species that cause in vivo double strand DNA damage is still a matter of debate for this class of compounds. It is possible that there may be a combination of two or more radical species which might cause DNA damage in a complex biological matrix. We also would like to consider that, a release of hydroxyl radical could itself generate a counterpart benzotriazinyl radical, which ultimately cause tumor cell damage (Scheme 3.9).
3.17 Experimental Section

**Materials.** Materials were of the highest purity available and were obtained from following sources: sodium phosphate, mannitol, xanthine, DMSO, and TLC plates from Aldrich Chemical Co. (Milwaukee, WI); NADPH, desferal, cytochrome P450 reductase, catalase, calf thymus DNA, and superoxide dismutase (SOD) from Sigma Chemical Co. (St. Louis, MO); xylene cyanol, bromophenol blue, formamide, and urea from United States Biochemical; T4 polynucleotide kinase from New England Biolabs; [γ- 32 P]-dATP from Perkin-Elmer Life Sciences; oligonucleotides were purchased from Integrated DNA Technologies; acrylamide and bisacrylamide from Roche Diagnostics;
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xanthine oxidase from Roche Diagnostics; agarose from Seakem; HPLC grade solvents (acetonitrile, methanol, ethanol, tert-butyl alcohol, ethyl acetate, hexane, and acetic acid) from Fischer (Pittsburgh, PA); ethidium bromide from Roche Molecular Biochemicals (Indianapolis, IN); Silica gel (0.04 0.063 mm pore size) for column chromatography from Merck. The plasmid pGL2BASIC was prepared using standard protocols.  

Tirapazamine (1, TPZ), 12 were synthesized according to literature methods.  

3.18 Cleavage of Supercoiled Plasmid DNA

In a DNA cleavage assay, a solution of 30 µL containing supercoiled plasmid DNA (1000 ng), NADPH (500 µM), cytochrome P450 reductase (1 mU), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM) were incubated with di-N-oxide (12 or TPZ, 250 µM). In all DNA damage reactions, individual components, except DNA, NADPH, and enzymes, were deoxygenated by using three cycles of freeze-pump-thaw in pyrex tubes and then torch-sealed under high vacuum. Sealed tubes were scored, and opened in a glove bag filled with argon, and used to prepare individual reactions. Enzymes, NADPH, and DNA were diluted with degassed water in the glove bag to prepare stock solutions. Reactions were initiated by adding cytochrome P450 reductase, then wrapped with aluminium foil to prevent exposure to light, and incubated for 3 h in the glove bag at room temperature (25 ºC). Following incubation, the reactions were stopped by adding 5 µL of 50% glycerol loading buffer, and the resulting mixture was loaded onto a 0.9% agarose gel. The gel was electrophoresed for approximately 2.5 h at 82 V in 1 X TAE buffer then stained in a solution of aqueous ethidium bromide (0.3 µg/mL) for 3 h. DNA in gel was visualized
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by UV-transillumination, and the amount of DNA in each band was quantified using an Alpha Innotech IS-1000 digital imaging system. DNA-cleavage assays containing radical scavengers were performed as described above with the exception that radical scavengers such as a methanol, ethanol, tert-butyl alcohol, DMSO, or mannitol (100 mM) were added to the reaction before addition of cytochrome P450 reductase. To prevent the background DNA damage from superoxide radical, superoxide dismutase, catalase and desferal were added to reactions.

3.19 Sequence Specificity of DNA Strand Cleavage by 12

A 30-base 2'-deoxyoligonucleotide was 5'-end labeled with $^{32}$P using [$\gamma$-$^{32}$ P] (5'-GTCACGTGCTGCAGACGACGTGCTGAGCCT-3') dATP and T4 polynucleotide kinase and was purified on a 20% denaturing polyacrylamide gel. The labeled single strand oligonucleotide was then annealed with its complimentary strand by heating the mixture to 90 °C sodium phosphate (pH 7, 20 mM) followed by slow cooling to room temperature overnight. DNA cleavage by 4 and 12 was performed in a dialysis chamber placed in deoxygenated aqueous buffer (three freeze-pump-thaw cycles) inside an inert atmosphere glovebag as described previously. The degassed reaction mixture containing duplex oligonucleotide (250,500 cpm), 4 (250 µM) or 12 (250 µM), sodium phosphate buffer (10 mM, pH 7.0), desferal (1 mM), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), NADPH (1 mM) and cytochrome P450 reductase (0.05 U/mL) in a total volume of 100 µL was pipetted into a Slide-A- Lyzer minimal unit (Pierce, 3000 MW cutoff). The dialysis unit was then placed with a floater into 2 mL of solution containing 1 (250 µM) or 5 (250 µM), NADPH (1 mM), sodium phosphate buffer (10 mM, pH 7.0),
and desferal (1 mM) and gently stirred for 16 h in a glovebag filled with argon, followed by the removal of DNA-containing solution from inside the dialysis unit. The assay shown in Figure 3.5H was carried out in an identical manner, except the assay contained 40 mM NaCl. The oligonucleotide was precipitated using 0.3 M sodium acetate, 70% ethanol (final concentration), and 5 µg of carrier DNA (herring sperm), and then washed three times with 80% ethanol-water. The resulting pellet was air-dried, the DNA fragments dissolved in formamide loading buffer, heated at 90 °C for 5 min, then immersed in ice water. An equal number of counts were loaded in each lane of 20% denaturing polyacrylamide gel. The gel was electrophoresed for 3h at 1200 V in 10× TBE buffer. The resolved fragments of DNA in the gel were visualized by phosphorimager analysis (Molecular ImagerFX, Imaging Screen-K, cat 170-7841, Bio-Rad, using Quantity One Version 4.2.3, Bio-Rad). Maxam-Gilbert A + G and G-reactions shown in Figure 3.5 were carried out according to the literature methods.\textsuperscript{20,38} DNA cleavage by iron EDTA/H2O2/ascorbate was performed according to the protocol of Tullius and co-workers.\textsuperscript{28}

**3.21 DNA Strand Cleavage by Hydroxyl Radical Generated by Anaerobic Photolysis of Hydrogen Peroxide**

The DNA duplex described above was dissolved in a solution containing 10 mM sodium phosphate (pH 7), 40 mM NaCl, 500 mM hydrogen peroxide and subjected to freeze pump thaw degassing to remove dissolved gases as described above. The mixture was subjected irradiation with UV light (Spectroline MODEL ENF-240C, 115 V, 60 Hz, 0.20 A, long wavelength 365 nm) in Pyrex tubes (6 mm i.d.) for 10 15 min at room
temperature (25 °C). The oligonucleotide was precipitated using 0.3 M sodium acetate, 70% ethanol (final concentration), and 5 µg of carrier DNA (herring sperm), and then washed three times with 80% ethanol water. The resulting pellet was air-dried, the DNA fragments resuspended in formamide loading buffer, heated at 90 °C for 5 min, then immersed in ice water. An equal number of counts were loaded in each lane of 20% denaturing polyacrylamide gel. The gel was electrophoresed for 3 hr 1200Vin10× TBE buffer. The resolved fragments of DNA in the gel were visualized by phosphorimager analysis (Molecular ImagerFX, Imaging Screen-K, cat 170 7841, Bio-Rad, using Quantity One Version 4.2.3, Bio-Rad). Sequencing lanes were prepared as described above.

References

Molecular Mechanism of DNA Damage by 3-Methyl-1,2,4-benzotriazine 1,4-di-N-oxide

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Chapter 3

Molecular Mechanism of DNA Damage by 3-Methyl-1,2,4-benzotriazine 1,4-di-N-oxide


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Chapter 3

Molecular Mechanism of DNA Damage by 3-Methyl-1,2,4-benzotriazine 1,4-di-N-oxide


4.1 Introduction

Myxin (1-hydroxy-6-methoxy-phenazine-5,10-dioxide (52), is a natural product, in the common class of phenazine N-oxides, which exhibit toxicity toward a wide spectrum of organisms including gram-positive and gram-negative bacteria, fungi, yeast, and algae.\textsuperscript{1-4} It was first isolated from the Sorangium species.\textsuperscript{1,4} In vivo assays of myxin, show inhibition of DNA biosynthesis and degradation of DNA in \textit{E. coli}.\textsuperscript{6,7} Myxin has a planar structure and contains structural similarities with acridine, anthracene, and other phenazine compounds, which makes myxin a classical non-covalent DNA binder.\textsuperscript{6-8}

Heterocyclic N-oxides are currently under various preclinical studies as potential anticancer candidates.\textsuperscript{9,10} Recently, Pacho’n et al. have reported a class of phenazine-N-
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oxide derivatives as hypoxia selective cytotoxic agents. They have done structure activity relationship studies with different cancer cell lines (Scheme 4.1), although the exact mechanism of their biological activities is not well defined.

Scheme 4.1: Structures of new phenazine dioxide derivatives as hypoxia selective cytotoxic agents

Tirapazamine is the lead compound in this class of heterocyclic N-oxides, and is currently undergoing phase I, II and II clinical trials for the treatment of various human cancers such as non-small lung cancers, and head and neck cancers. There is an ongoing effort to make new analogs of tirapazamine for optimum drug efficacy. For example, TPZ was combined with a DNA intercalator, and the action of drug has been improved. Thus, myxin and its heterocyclic N-oxide analogs with moderate DNA binding abilities, may have good drug potency in the preclinical studies.

To the best of our knowledge, there is no single report on any direct evidence that 52 can cause DNA strand cleavage under bioreductive activation and hypoxic conditions. Previously, our group has worked with myxin extensively, and shown that a methylated derivative, 1,6 dimethoxyphenazine-5,10 dioxide (53), is capable of cleaving DNA when
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enzymatically activated in vitro. In this chapter, we briefly discuss the possible mechanism of DNA damage by 52 and 53 and report the sequencing gel analysis of a 30 base pair oligonucleotide.

![Structures of natural product Myxin and its synthetic analog methul-myxin](image)

**Scheme 4.2:** Structures of natural product Myxin and its synthetic analog methul-myxin

## 4.2 Redox-activated Hypoxia Selective DNA Cleavage by 52 and 53

Compound 52 and 53, were synthesized by literature procedure\(^{15}\), followed by \(N\)-oxidation using \(m\)-chloroperbenzoic acid. Our group has found, that upon bioreductive activation, and hypoxic conditions both 4a and 4b induce DNA strand cleavage. NADPH:cytochrome P450 reductase or xanthine/xanthine oxidase enzyme systems were utilized for the one-electron reductive activation of this class of molecules, because these enzyme systems are widely used for in-vivo and in-vitro studies respectively.\(^{16,17}\) To establish the hypoxic conditions, molecular oxygen was removed from the stock solutions by three cycles of freeze-pump-thaw degassing, and the assay mixtures were prepared and incubated in an inert atmosphere glovebag. Reactions were incubated for 4-6 hours. Other additives such as catalase, superoxide dismutase, and desferal combination were used to inhibit molecular oxygen mediated DNA cleavage. When the compounds were incubated either alone or with xanthine alone or with the complete enzymatic reducing system under aerobic conditions, no significant amounts DNA strand cleavage are observed, except myxin alone where the DNA cleavage was observed in the oxic
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conditions.\textsuperscript{17a} In contrast, methyl myxin did not show any significant DNA cleavage under aerobic conditions, which is typical of hypoxia selective cytotoxic agents. DNA strand cleavage by myxin and methyl myxin under aerobic conditions may be fundamentally interesting to us as a structure activity point of view, because a single atomic change in a molecule can alter its hypoxia selectivity. The mechanism of observed DNA damage by myxin itself, under oxic conditions, is still under investigation.

Presence of classical radical scavengers like ethanol, methanol, t-butanol, DMSO, and mannitol significantly inhibited DNA cleavage. Together these results demonstrate that DNA cleavage by myxin and methylmyxin is redox activated, and DNA cleavage could be mediated by the small diffusible hydroxyl radical species, similar to TPZ.

4.3 Redox Activated in vitro Metabolism Studies of 52 and 53

Our group and others have extensively studied on the metabolism of TPZ and various TPZ analogs.\textsuperscript{15,16,17,18} In the presence of NADPH:cytochrome P450 reductase or xanthine:xanthine oxidase, TPZ yields 3-amino-1,2,4-benzotriazine-1-oxide as the major metabolite. Identification of metabolites is an important part of in vitro biochemical studies to understand the mechanism of DNA strand cleavage by TPZ and other various heterocyclic N-oxides.\textsuperscript{15} Previous work from our group examined the products generated by one-electron reductive activation of myxin and methylmyxin by the enzyme xanthine:xanthine oxidase under anaerobic conditions. In vitro metabolism of methylmyxin yielded 1-N-oxide as the major and no-oxide as the minor metabolite. The major metabolite from myxin metabolism reaction was found as mono-N-oxide 1-hydroxy-6 methoxyphenazine 10-N-oxide (Chowdhury`s thesis data, chapter 5, p-206).
4.4 Sequence Specificity of DNA strand cleavage by 52 and 53

We have suggested bioreductively activated benzotriazine N-oxides get metabolized with the release of an hydroxyl radical. It is clear that the benzotriazine N-oxides cause DNA strand cleavage at every nucleotide, but with a slight preference for cleavage at purine residues. We hypothesize if myxin and methylmyxin release hydroxyl radical, the DNA cleavage of these compounds should be sequence independent. To test our hypothesis, we examined the sequence-specificity of DNA strand cleavage by myxin and its methylated analog. In these experiments, a 30-base pair, 5′-32P-labeled oligodeoxynucleotide duplex, was treated with 52 and 53 and the xanthine:xanthine oxidase system under anoxic conditions. Following the DNA-damage reaction, the 32P-labeled DNA fragments were resolved on a denaturing polyacrylamide sequencing gel and visualized by phosphorimager analysis. The relevant lanes from the gel are displayed as densitometry traces, in which each cleavage band appears as a peak (Figure 4.1). We find that DNA strand cleavage by reductively activated methylmyxin, occurs at every base pair in the duplex. The pattern of sequence-independent DNA strand cleavage caused by methylmyxin closely resembles that generated by myxin (Figure 4.1C), and is generally characteristic of a highly oxidizing, small, diffusible species such as hydroxyl radical. We compared the DNA strand cleavage of myxin and methyl myxin to that of an iron-EDTA and a Maxam Gilbert G reaction systems which generates hydroxyl radical mediated oxidative damage and guanine specific strand cleavage respectively (or a functionally equivalent species). Comparison with the iron-EDTA cleavage lane reveals both myxin and methylmyxin display
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Figure 4.1: (A-D) Comparison of DNA-cleavage patterns generated by enzymatically activated myxin methyl myxin,, Maxam-Gilbert G reaction and iron-EDTA. (A) methylmyxin activated by X/XO enzyme system under anaerobic conditions, and compared with Maxam-Gilbert G reaction lane (B) methylmyxin was activated by X/XO enzyme system under anaerobic conditions, and compared with the hydroxyl radical-generating Fe-EDTA system and, (C) myxin was activated by X/XO enzyme system under anaerobic conditions, and compared with the methylmyxin reaction lane under the same conditions. DNA cleavage reactions were performed on a 30 base pair 5′- 32P-labeled oligonucleotide. Densitometer scans are from a portion of a 20% denaturing polyacrylamide gel and show the relative intensity of DNA cleavage at each base position. Lanes were loaded with equal amounts (cpm) of labeled DNA and are plotted on the same vertical scale so they can be compared.
sequence independent but little preference for cleavage at purine residues in the DNA duplex. From our previous data and experience with N-oxide heterocyclic compounds, we believe that myxin and methyl myxin cleave DNA by a release of hydroxyl radical. Then, why is the DNA sequence specificity by these N-oxides not a mirror image of hydroxyl radical? In case of benzotriazine-di-oxides, we observed the similar sequence specificity pattern. In that case, generation of the benzotriazinyl radical could be a minor process, responsible for this little purine specificity. In case of myxin and methylmyxin most probably, no benzotriazinyl type radical via dehydration mechanism pathway, is possible. However, an intermediate hydroxyl radical adduct with deoxypurine may undergo a complex secondary reaction with N-oxides, or other metabolites, which ultimately may lead to the generation of the extra strand cleavage in our experiments (chapter 3). It is also known that oxidation-induced sugar damage affords sites leading to frank strand scission or heat-labile sites. However, we have no direct evidence which explains the existence of sequence specificity by this class of N-oxide compounds under our reaction conditions, but we would like to consider that myxin and methylmyxin cause DNA strand cleavage primarily via production of hydroxyl radicals (Scheme 4.3).

4.5 Proposed Mechanism of DNA Damage by Myxin and Memyxin

N-oxide class of DNA damaging agents share a common motif, an N-oxide, which is the key functional group for their biological activity and in vitro DNA damaging ability. From our in-depth understanding of TPZ chemistry under bioreductive activation and low oxygen concentrations, we envisioned that Myxin and Methylmyxin would behave as TPZ. Nagai et al. proposed that 9-substituted phenazine-N-oxides, upon one electron
reduce sources such as NADPH and DTT (dithiotritol) might cause DNA damage through the generation of hydroxyl radical under anaerobic conditions. All together, the proposed mechanism (scheme 4.3) of DNA damage by 52 and 53 under low oxygen conditions and reductive activation such as NADPH/cytochrome P450 redcutase or xanthine/xanthine oxidase is reasonable.\(^6\)

\[ \text{Scheme 4.3: Proposed mechanism of DNA damage by 53 under reductive activation} \]

4.6 Aerobic DNA Cleavage by 52 Without X/XO as an Activating System.

All reactions contained 5'-\(^{32}\)P labeled 30 mer oligonucleotide duplex and sodium phosphate buffer (10 mM, pH 7.0). The reactions were incubated for 24 h under oxic conditions at 24 °C, followed by 20% polyacrylamide gel electrophoresis. Myxin concentration was always kept at 500 µM. All the reactions were kept in the dark. SOD (10-50 µg/ml) only was used in one of the sequencing gel lanes. Myxin was able to cause DNA cleavage without any bioreductive activation under oxic condition. Under these conditions, myxin did cleave DNA at every base pair. When superoxide dismutase (SOD) was used, no DNA cleavage was observed. From the previous unpublished agarose gel
data (Chowdhury, unpublished data) it was found that catalase and desferal independently
could not stop DNA cleavage by myxin. If superoxide radical is directly responsible for
the oxidative DNA damage, then addition of SOD should induce more DNA cleavage
through the production of hydrogen peroxide. However, it was found that SOD inhibits
DNA cleavage mediated by myxin under oxic condition (Figure 4.2). However, superoxide may have a direct significant role on deactivating myxin. The mechanism of
Myxin activation under aerobic conditions is not fully understood yet.

Figure 4.2: SOD mediated inhibition of DNA cleavage by 52 under aerobic condition. Cleavage of 5'-32P
labeled 30-mer oligonucleotide duplex by myxin under aerobic condition without any activating systems.
All the reactions contained 5'-32P labeled 30-mer oligonucleotide duplex (250,000 cpm) in sodium
phosphate buffer (10 mM pH 7). The reaction were incubated for 24 h under aerobic conditions at room
temperature, followed by 20% gel electrophoresis. Lane 1, DNA alone, Lane 2, DNA+ myxin (500 µM) +
buffer, Lane 3, DNA + myxin (500 µM) + SOD (10 µg/mL)

4.7 Proposed Mechanism of DNA Cleavage by 52 and DNA Cleavage Inhibition
Mediated by SOD Without Bioreductive Activation Under Aerobic Conditions
. When the compounds are incubated with the either alone or with xanthine alone, no
significant amounts DNA strand cleavage are observed, except with myxin alone where
DNA cleavage is observed under oxic conditions. In contrast, methyl myxin did not show any significant DNA cleavage under aerobic conditions, which is typical as hypoxia selective cytotoxic agents. The mechanism of observed DNA damage by myxin itself under oxic condition is still under investigation. (Scheme 4.3)

**Scheme 4.4:** Proposed mechanism of DNA damage by myxin under aerobic conditions

4.8 Conclusion:

From our studies and current understanding of N-oxide hetercycles, we have presented the first time that myxin, a natural product, and its methylated analog can induce oxidative DNA damage upon one electron reductive activation. DNA damage stems through the generation of hydroxyl radical. methylmyxin is hypoxia selective DNA
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damaging but myxin: This is very interesting phenomenon for two similar compounds/one methoxy group makes the difference

4.9 Experimental

Sequence Specificity Experiments:

A 30 base pair oligonucleotide with a sequence of 5'-GTCACGTGCTGCAGACGACGTGCTGAGCCT-3' was 5'-end labeled with $^{32}$P using [$\gamma$-$^{32}$P] dATP and T4 polynucleotide kinase and was purified on a 20% denaturing polyacrylamide gel. The labeled single strand oligonucleotide was then annealed with its complimentary strand by heating the mixture to 90 °C followed by slow cooling to room temperature for overnight. For this sequence specificity experiment, oligonucleotide, NADPH, cytochrome P450 reductase, 53, and 52 were placed inside a dialysis bag and suspended in a sodium phosphate buffer solution containing the enzyme substrate and compounds. In this way the steady concentration of xanthine and 52, 53 can be maintained throughout the reaction time and the amount of cleavage is moderately increased. All the reactions were kept in the dark. Cleavage reactions by 53 and 52 were run in deoxygenated aqueous buffer (three freeze-pump-thaw cycles) inside an inert atmosphere glove bag. Degassed reaction mixture containing the duplex oligonucleotide (250,000 cpm), 53 and 52 each (500 μM), sodium phosphate buffer (10 mM, pH 7.0), desferal (1 mM), catalase (100 µg/mL), superoxide dismutase (10 µg/mL), Xanthine (500 μM) and Xanthine oxidase (1 U/mL) in a total volume of 100 μL was pipetted into a Slide-A-Lyzer minimal unit (Pierce, 3000 MW cutoff). The dialysis unit was then placed with a floater into 2 mL of solution containing myxin and methyl myxin each (500 μM),
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sodium phosphate buffer (10 mM, pH 7.0), and desferal (1 mM) and gently stirred for 16 h in a glove bag filled with argon, followed by the removal of DNA from the dialysis unit. The oligonucleotide was precipitated using 0.3 M sodium acetate, 70% ethanol (final concentration) and 5 µg of carrier DNA (herring sperm), and then washed three times with 80% ethanol/water. The dried oligonucleotide fragments were dissolved in formamide loading buffer, heated at 90 ºC for 5 min, then immersed in ice water and equal number of counts were loaded in each lane of 20% denaturing polyacrylamide gel. The gel was electrophoresed for 3 h at 1200 V in 10×TBE buffer. The DNA fragments in the gel were visualized by phosphorimager analysis (Molecular Imager®FX, Imaging Screen-K, cat 170-7841, Bio-Rad, using Quantity One® Version 4.2.3, Bio-Rad). The Maxam-Gilbert A + G and G-reactions were performed following standard protocols. DNA cleavage by iron EDTA/H2O2/ascorbate was carried out as described by Tullius and co-workers.22
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Investigation of Oxygen Mimetic Properties of Nitrous Oxide

5.1 Introduction

Nitrous oxide (N\textsubscript{2}O, 57) is known as laughing gas, a substance used as anesthetic during surgery, radiation therapy and dentistry. N\textsubscript{2}O is also known to cause infertility, spontaneous abortion, and birth defects, and other disease states.\textsuperscript{1-4} Nitrous oxide is a known trapping agent for solvated electrons. In radiolysis of aqueous solution nitrous oxide is routinely used to convert solvated electrons into hydroxyl radicals.\textsuperscript{5,6} N\textsubscript{2}O was assumed to be inert to carbon centered radicals. However, it has been demonstrated by Yong-Tae and Kwang-Wook Kim that some carbon centered radicals are efficiently trapped by nitrous oxide. It was shown that the formation of biphenyl from benzoyl
peroxide precursor is inhibited by the presence of nitrous oxide, similar to that seen for molecular oxygen (Scheme 5.1).\textsuperscript{7,8} Thus, nitrous oxide can act as moderate radical quencher.

Scheme 5.1: Radical trapping experiment by Nitrous oxide

In radiation therapy, reactive free radicals are generated in vivo, generally as hydroxyl radicals which are known to cause, protein, lipid, and DNA oxidation.\textsuperscript{9-11} Highly reactive, small, diffusible hydroxyl radical can generate \textit{insitu} deoxyribonucleotide radicals, which undergo further oxidation, and a strand cleavage.\textsuperscript{12} A free radical, a chemical species, is capable of independent existence possessing one or more unpaired electrons. Biological free radicals thus are highly unstable molecules which have electrons available to react with various organic substances.\textsuperscript{6} Free radicals react with key organic substances such as lipids, proteins, and DNA. Oxidation of these bio-molecules leads to disruption of normal functions and may contribute to a variety of disease states, especially in when the damage is done in the DNA.\textsuperscript{13,14}

This year, it has been shown that occupational exposure which at least is 180 mg/m\textsuperscript{3} to anesthetic such as N\textsubscript{2}O has been suggested to increase risk of genetic damage, and ultimately DNA damage is associated with the longer exposure of nitrous oxide.\textsuperscript{15,16}
5.2. Can Nitrous Oxide Trap Deoxyribonucleotide Radical, and Lead to DNA Strand Cleavage?

The compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, 1) is hypoxia-selective antitumor agent which is currently undergoing phase II, and III clinical trials for the treatment of various human cancers. Tirapazamine selectively cause DNA damage in hypoxic tumor cells. Under bioreductive activation, tirapazamine release one more reactive radicals, such as hydroxyl or benzotriazinyl radicals, which ultimately cause strand cleavage. These radicals abstract hydrogen from the sugar backbone of DNA and produce deoxyribonucleotide radicals. Deoxyribonucleotide radicals are important reactive intermediates in a variety of DNA damage processes.

Tirapazamine is found to react with sugar radicals and cause oxidative DNA damage. For example, C1’ radical in DNA is shown to react with TPZ, and the trapping rate of C1’-DNA radical by tirapazamine is \( \sim 2.8 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \). The authentic C1’-DNA radical was generated by photolysis of a t-butyl ketone radical precursor (Scheme 5.1).

\[
\begin{align*}
\text{58} & \quad \xrightarrow{\text{hu}} \quad \text{59}
\end{align*}
\]

Scheme 5.1: Formation of deoxynucleoside radical from 2’- deoxyuridine-1’-yl

Based on the observations of Yong-Tae et al., and the fact that \( \text{N}_2\text{O} \) is a widely used additive in the radiation chemistry of DNA, we thought it would be fundamentally interesting to see whether \( \text{N}_2\text{O} \) can act as surrogate for molecular oxygen and react with a C1’ DNA radical. It is worth mentioning that \( \text{N}_2\text{O} \) shares a part of structural unit of tirapazamine, which might lead some interesting DNA damaging chemistry.
5.3 The Competition Experiment

In an experiment originally reported by Greenberg et. al reaction of an authentic C1’ DNA radical with O₂ results in the formation of ribonolactoctone, which upon base treatment results DNA strand cleavage. Presence of thiol however quench the DNA sugar radical as protective mechanism. Thus, in the presence of thiols, and O₂ a competition occurs for the DNA radical which can be quantified from the amount of cleaved and uncleaved DNA (Scheme 5.3). The amount of DNA cleavage can be determined by PAGE analysis (equation 1).

\[
\frac{\text{cleaved}}{\text{uncleaved}} = \frac{k_{\text{O}_2} [\text{O}_2]}{k_{\text{RSH}} [\text{RSH}]} \quad (1)
\]

In case of the nucleoside radical, reaction with O₂ and thiols results in the formation of ribonolactone and nucleoside respectively that can be quantified using HPLC. ²⁵,²⁶ In this HPLC study, the desired products (Scheme 5.1.2) were quantified in order to calculate the trapping rate constant.

Scheme 5.3: Formation of deoxynucleoside radical from 2’- deoxyuridine-1’-yl radical 59
Investigation of Oxygen Mimetic Properties of Nitrous Oxide

Scheme 5.4: Possible products from the nucleoside radical reaction with N₂O are shown below.

Based on the observation of Yong-Tae et al. we propose the reaction of N₂O with the C1’ radical will result in the formation of ribonolactone as shown in Scheme 5.5.

Scheme 5.5: Proposed mechanism of reaction of N₂O with the C1’ radical.

5.4 Discussion and Conclusion

At first, we proposed that nitrous oxide might play a subtle but significant affects inducing DNA damage. We secondly proposed that under highly oxidative stress conditions, N₂O might oxidize DNA and cause single strand cleavage. We also proposed that nitrous oxide can act as oxygen surrogate and responsible for DNA damage in acute hypoxia.
Investigation of Oxygen Mimetic Properties of Nitrous Oxide

We have evidence that under low oxygen condition nitrous oxide induces DNA damage. In this chapter we report preliminary results of DNA damage induced by Nitrous oxide. We declare that there is no conflict of interest on this study. Here we report preliminary results and future experiments regarding the interaction of N₂O with a C1’ DNA radical. We have presented evidence that nitrous oxide does interact with DNA radicals. Comparing the kinetics of trapping DNA radicals by thiols and N₂O, we have shown that the rate constant for the reaction of nitrous oxide with the C1’ radical is in the order of $10^5$. Irradiation of freeze-pump-thaw degassed samples of [5’-32P] in the presence of dissolved nitrous oxide produced yields of alkaline labile lesions that depended inversely on the concentration of β-mercaptoethanol (Figure 5.2). The rate constant is comparable to C1’ radical trapping reaction of Tirapazamine.\(^{24,25}\) This radical trapping rate of reaction of nitrous oxide is slower than that for trapping by O₂, but comparable to double-stranded DNA radical quenching reaction rate by thiol, suggests that low millimolar concentrations of N₂O can compete with thiol for a DNA radical reaction. Further investigations regarding the yield of ribonolactone with the aid of GC/MS spectroscopy and isotope labeling experiments to understand whether there is any oxygen donation from the nitrous oxide or from water are underway. We have tried our best to remove and exclude molecular oxygen though some of the reaction mixtures may contain lower amount of dissolved oxygen. We still have to consider the effect of oxygen in the reaction mixture. The rate constant of oxygen for trapping a carbon radical is a fast process which is $1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$. When we purged with N₂ or argon through a solution for about 2-5 minutes, the O₂ concentration goes down to around 1 µM. We removed molecular oxygen by 3 to 4 cycles of freeze-pump-thaw, and then degassed with either N₂ or N₂O.
Investigation of Oxygen Mimetic Properties of Nitrous Oxide

If we consider presence of 1 μM or low micromolar of oxygen in the reaction mixture, we still get low trapping efficiency compared to nitrous oxide. Although, presence of 7-10 μM of molecular O₂ can provide trapping rate constant similar to N₂O under our reaction conditions. There are many ways molecular oxygen can get in the reaction mixture. To monitor the concentration of oxygen during the reactions, we would use an oxygen probe as a part of our future goal. However, under nitrogen atmosphere and in absence of nitrous oxide, photolysis reaction followed by base workup did not produce significant strand cleavage. To this end, we conclude that N₂O, a well known anesthetic, acts as surrogate for molecular oxygen and react with a double-stranded and C1’ nucleoside radical with a rate constant of $1.22 \times 10^5 \text{M}^{-1}\text{S}^{-1}$ and $1.77 \times 10^8 \text{M}^{-1}\text{S}^{-1}$ respectively to yield base labile DNA strand cleavage.
5.5 Experimental

We performed two different poly acrylamide gel electrophoresis and High Pressure Liquid Chromatography to determine the trapping ability of N\textsubscript{2}O.

Materials were of the highest purity available and were obtained from following sources: xylene cyanol, bromophenol blue, formamide, and urea from United States Biochemical; T4 polynucleotide kinase from New England Biolabs; [\gamma-32P]-dATP from Perkin-Elmer Life Sciences; acrylamide and bisacrylamide from Roche Diagnostics; HPLC grade solvents (acetonitrile, water, methanol) from Fischer; 32P-labeling was carried out with T4 polynucleotide kinase following standard procedures. Oligonucleotides were sequenced using a reaction specific for 2’-deoxyadenosine.\textsuperscript{24}

Pyrex photolysis tubes were loaded with the appropriate reagents; the samples were degassed with three freeze-pump-thaw cycles (5 min each) and the tubes sealed under vacuum. Photolyses were carried out for 20 min in Pyrex tubes (6 mm i.d.) using a Rayonet photoreactor equipped with 16 lamps (\lambda \text{max} 350 nm).

5.6 Investigation of the DNA-Cleaving Properties of Nitrous Oxide: Study by Ploy Acrylamide Gel Electrophoresis Experiment

In this experiment, we wanted to observe whether nitrous oxide can compete with thiols for reaction at the DNA C1’ radical. After the generation of C1’ radical, it can be
quenched either by thiol to yield repaired DNA or reacted with nitrous oxide to generate a labile lesion (Scheme 5.1.3). Upon base workup, this labile lesions in DNA are being observed by gel electrophoresis as a DNA strand break. Observed strand cleavage is a measure of the relative rates for trapping nitrous oxide and trapping by various concentrations of thiols. Considering the rate constants for the reaction of thiol and C1’-nucleotide radical in single- and doublestranded DNA are as follow $4.4 \times 10^6$ and $1.8 \times 10^6$ M$^{-1}$ s$^{-1}$, we could calculate the overall rate constant for the radical trapping reaction of nitrous oxide with the C1’-nucleotide radical. For this experimental part, double-stranded 5’-32P labeled oligonucleotide was irradiated under anaerobic conditions in the presence of known concentration of nitrous oxide and varying known concentrations of thiol. The rate constants for the trapping reaction was calculated from the slope obtained by plotting yields of strand cleavage versus varying thiol concentration in molarity. (Figure 1). Rate constants for the reaction of nitrous oxide with the C1’-radical is shown in Table 1.

**Figure 5.3:** Determination of the effect of β-mercaptoethanol on the formation of alkaline-labile lesions from 2’-deoxyuridin-1’-yl in double-stranded DNA in the presence of nitrous oxide; Lane 1-7 reactions in presence of fixed nitrous oxide and varying thiol concentrations, lane 6-14 reactions in presence of nitrogen gas only and thiol.
Conditions for the assays

NaCl (100 mM), NaP buffer (10 mM), N₂O saturated ~ 30.66 mM, thiol (100 µM- 100 mM), total reaction volume 100 µL.

Figure 5.4: Determination of the effect of β-mercaptoethanol on the formation of alkaline-labile lesions from 2’-deoxyuridin-1’-yl in double-stranded DNA in the presence of nitrous oxide
5.7 Calculation of rate constants for the trapping of a C1’-Radical in DNA by nitrous oxide

$k_{T(RSH)}$ for the double-stranded DNA is $1.8 \times 10^6 \text{M}^{-1}\text{S}^{-1}$ (Hwang et al. *Biochemistry*, *Vol. 38, No. 43, 1999*)

\[
k_{N2O} = \text{slope} \times 1.8 \times 10^6 \times 1000 \quad (\text{slope} = 0.002)
\]

\[= 29.6 \times 10^5 \text{M}^{-1}\text{S}^{-1}\]

Calculation of $k_{N2O}$ from HPLC data

\[
\frac{[dU]}{[U]} = \frac{k_{RSH}[RSH]}{k_{N2O}[N_2O]}
\]

$k_{T(RSH)}$ for the double-stranded DNA is $4.6 \times 10^6 \text{M}^{-1}\text{S}^{-1}$

\[m = \text{slope} = 8.38 = \frac{k_{RSH}}{k_{N2O}[N_2O]} \quad k_{N2O} = 1.77 \times 10^8 \text{M}^{-1}\text{S}^{-1}\]
5.8 HPLC Trace of N₂O + RSH Reaction

**N₂O Efficiently Competes with Thiol for the C1’ DNA Radical**

N₂O causes a ~5 fold increase in the formation of Uracil over N₂.

**Relative Formation of Uracil and Deoxyuridine**

Presence of N₂O causes an increase in the formation of uracil and a decrease in the formation of deoxyuridine-clear indication that N₂O can compete with thiol for the C1’ radical.

**Figure 5.5:** Relative trapping efficiencies of N₂O and Thiol with photolytically generated C1’ radical
### Table 5.2: Deoxyuridine/Uracil areas ratios and plots. Three sets of reactions were done and data were taken to get an idea of N$_2$O kinetics with the precursor radical.

<table>
<thead>
<tr>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
<th>average</th>
<th>std dev</th>
</tr>
</thead>
<tbody>
<tr>
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<td>dU</td>
<td>dU/U</td>
<td>U</td>
<td>dU</td>
</tr>
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</tr>
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<td>139371</td>
</tr>
<tr>
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<td>15.78936</td>
<td>3947</td>
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<td>4679</td>
<td>365777</td>
</tr>
<tr>
<td>5678</td>
<td>1131429</td>
<td>199.2654</td>
<td>3807</td>
<td>321860</td>
</tr>
</tbody>
</table>

**Figure 5.6:** Graph plotted in terms of ratio of peak areas of Uracil and Deoxyuridine. dU/U was plotted against varying thiol including average of all three experiments.
5.9 Experimental Section for HPLC Method.

Chemicals were purchased from the following suppliers and were the highest purity available: xylene cyanol, bromophenol blue, formamide, and urea from United States Biochemical; T4 polynucleotide kinase from New England Biolabs; $[\gamma$- 32 P]-dATP from Perkin-Elmer Life Sciences; oligonucleotides were purchased from Integrated DNA Technologies; acrylamide and bisacrylamide from Roche Diagnostics; Uracil (98%), 3-Mercapto-1-propanol (95%), Aldrich Chemical Co.; 2′-Deoxyuridine (99-100%), Sigma Chemical Co.; HPLC grade acetonitrile and water, Fisher. Pyrex brand standard borosilicate glass tubing was purchased from Ace. Glass. Water for HPLC and reactions was distilled, deionized, and glass redistilled.

General Procedure for the detection of deoxygenated products in the reaction of Nitrous oxide with 2′-deoxyuridine-1′-yl. In a typical assay (final volume 200 µL), a Pyrex tube (i.d. 12mm,) containing sodium phosphate buffer (10 mM, pH 7.0), 50 µM of 2′-deoxyuridine-1′-yl, 3-mercapto-1-propanol (1 mM to 15 mM), was freeze-pump-thaw degassed (3 times, each time about 5 minutes), followed by purging of N₂O for 20 minutes and then photolysed by employing a medium pressure mercury arc 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, 5µM pore size, 25 cm length, 4.6 mm i.d.) eluted with; Mobile phase A: Water, Mobile Phase B: Acetonitrile, gradient starts from
96% water, at 8 min it reaches 25% B at flow rate of 0.9 mL/min. The products were monitored by UV detection (260 nm).

The deoxygenated products were identified by comparison of their retention times to those of standards and confirmed by co-injection experiments.

**Figure 5.7:** HPLC analysis of products arising from the reaction of Nitrous oxide with 2′-deoxyuridine-1′-yl under anaerobic conditions. The resulting mixture was analyzed by reverse-phase HPLC, as described in the Experimental Section.
Investigation of Oxygen Mimetic Properties of Nitrous Oxide

Quantification of Base Labile DNA Lesions: A 2′-deoxyoligonucleotide GTCACGTGCTGC-∗GACGACGTGCTGAGCCT-3′ was P3′-end labeled with P using [γ- P] dATP and T4 polynucleotide kinase and was purified on a 20% denaturing polyacrylamide gel. The labeled single strand oligonucleotide was then annealed with its complimentary strand by heating the mixture to 90 °C sodium phosphate (pH 7, 20 mM) followed by slow cooling to room temperature overnight. The DNA used for this assay is the C1′ radical precursor provided by Dr. Greenberg. The reaction mixtures were freeze-pump-thaw degassed (3 times) in Pyrex tubes and then purged with either N2O or N2 followed by photolysis for 20 mins. Typically, [5′-32P] in phosphate buffer (pH 7.0, 10 mM), NaCl (100 mM), and the appropriate amount of β-mercaptoethanol (100 µM- 100 mM) were loaded in Pyrex tubes, then it was purged with N2O and the mixture was photolyzed for 20 min. Samples were then transferred to eppendorf tubes. The glass tubes were washed with 50 µL of water. The DNA was precipitated with NaOAc/EtOH, washing with 80% EtOH. The DNA was resuspended in aqueous piperidine (100 µL, 1M) and held at 95 °C for 20 min. The samples were lyophilized, resuspended in H2O, and lyophilized three times (each time 100 µL of HPLC H2O). The resulting pellet was air-dried, the DNA fragments dissolved in formamide loading buffer, heated at 50 °C for 5 min, then immersed in ice water. An equal number of counts were loaded in each lane of 20% denaturing polyacrylamide gel. The gel was electrophoresed for 3 h at 1200 V in 1× TBE buffer. The resolved fragments of DNA in the gel were visualized by phosphorimager analysis (Molecular ImagerFX, Imaging Screen-K, cat 170-7841, Bio-Rad, using Quantity One Version 4.2.3, Bio-Rad). Finally the DNA was dissolved in
loading buffer, denatured and loaded into a 20% denaturing polyacrylamide gel. It was first electrophoresed for 2 hrs at 700V and then for 3hrs at 1200 V.
Investigation of Oxygen Mimetic Properties of Nitrous Oxide

References


http://www.aapd.org/publications/brochures/nitrous.asp


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Investigation of Oxygen Mimetic Properties of Nitrous Oxide


18. Le, Quynh-Thu X.; Moon, James; Redman, Mary; Williamson, Stephen K.; Lara, Primo N., Jr; Goldberg, Zelanna; Gaspar, Laurie E.; Crowley, John J.; Moore, Dennis F., Jr.; Gandara, David R. *Journal of Clinical Oncology*, 2009, 27, 3014-3019


6.1 Introduction

Tirapazamine (TPZ, 4) is a promising antitumor agent that selectively causes oxidative DNA damage, which in due course, triggers cell death in the hypoxic cells found in solid tumors. TPZ is a promising bioreductive prodrug, currently in clinical trials phase III.\textsuperscript{1} Ubiquitous one-electron reductases such as cytochrome P450 reductase, xanthine oxidase, and other mammalian reductases, inject an electron into this molecule generate a TPZ radical anion, which is in equilibrium with its neutral radical (Scheme 6.1). Under aerobic conditions the compound undergoes relatively harmless redox cycling.\textsuperscript{2,7} On the other hand, under hypoxic conditions, the neutral radical undergoes elimination of a water or homolysis to generate benzotriazinyl or hydroxyl radical intermediate as the ultimate DNA damaging species.\textsuperscript{8-13, 14,15} The major drug metabolite
generated by hypoxic metabolism of TPZ is the 3-amino 1,2,4 benzotriazine 1-N-oxide (3) (Scheme 6.1.1).\(^\text{16,17,18}\)

\[
\text{4} 
\xrightarrow{1\text{e}^- + \text{H}^+}
\begin{array}{c}
\text{4a} \\
\text{4b} \\
\hline
\text{3}
\end{array}
\]

**Scheme 6.1:** Major drug metabolite generated by hypoxic metabolism of TPZ is the 3-amino 1,2,4 benzotriazine 1-N-oxide

Over a decade, we have been working with benzotriazine compounds, and we noticed that under regular UV lamp with longer wavelength, the major metabolite of TPZ gives bright green fluorescent color on the TLC plate. We found that this major metabolite displays green fluorescence (Em = 513 nm, in 50 mM of NaP buffer pH 7), when excited with 415 nm light. Interestingly, the parent drug and its minor metabolite 3-amino 1,2,4 benzotriazine are nonfluorescent. (Figure 6.1.1).

![Figure 6.1: TLC plate under UV lamp with 1-N-oxide and di-oxide](image)

The fortuitous fluorescence of the metabolite generated by hypoxic metabolism of antitumor agent TPZ presents an opportunity for the development of molecular tools for
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**Toward Fluorescent Probes of Tumor Hypoxia Based on 1,2,4 Benzotriazine-1-N-oxide Scaffold**

the detection of hypoxia in biological systems. In recent years, there has been increasing interest in the development of fluorescent molecules for the characterization of important biological processes \(^{19}\), but there are currently no commercially available probes for the direct fluorescent detection of hypoxia using small molecules. Fluorescence methods offer practical advantages with their sensitivity and the widespread availability of the instrumentation used to detect fluorescent compounds in biological systems. There may be a growing need for new tools to detect hypoxia in biological systems. Over a few decades, research on the chemistry and biology of hypoxia is growing which is depicted in Figure 6.2.

![Figure 6.2: How is Hypoxia Important These Days](image)

For example, it has been recently established that hypoxia plays an important role in cancer biology \(^{19,20}\) and the hypoxic environment may select for cells that are incapable of undergoing apoptosis. \(^{21}\) In addition, there is a recent speculation that cancer stem cells thought to be responsible for metastases are upregulated in the hypoxic niche of tumor. \(^{22}\)

Thus, detection of tumor hypoxia could eventually become an important routine aspect in the clinical characterization of human cancers. It is worth mentioning that hypoxia may
related to ischemic stroke, myocardial infarction, arthritis, the lung lesions of TB patients, and in inflammation.\textsuperscript{23,24,25}

TPZ is the most known bioreductive prodrug, which exploits hypoxia, and is effectively metabolized only under hypoxic conditions. This classical selection may be beneficial to serve as an effective probe for hypoxia in biological systems. From our observation, the 1-\textit{N}-oxide byproduct generated by the metabolism of TPZ under hypoxic conditions displays modest fluorescence properties. The extinction coefficient at the absorbance maxima is 4400 M\textsuperscript{-1} cm\textsuperscript{-1}, and the quantum yield is 0.12 in acetonitrile.\textsuperscript{26} Thus, 1-\textit{N}-oxide of TPZ yields a brightness of around 500, which is not comparable to commercially available agents used for imaging biological samples.\textsuperscript{26a} Structural modification on a parent fluorescent molecule is always a challenging task, because fluorescence properties are sensitive to structural changes. Thus, as a preliminary investigation, we set out to characterize a series of known analogues of TPZ and other heterocycles from our chemical library that might further inspire us to design brighter metabolites which could serve as fluorescent markers of hypoxia in biological systems. Herein, fluorescence properties of available 3-amino-1,2,4-benzotriazine-1-\textit{N}-oxides from our lab were characterized and were evaluated.

\section*{6.2 General Preparation of 3-Amino-1,2,4-benzotriazine 1-\textit{N}-oxide Derivatives}

Substituted analogues of TPZ typically could behave in a manner similar to the parent compound, undergoing hypoxia-selective metabolism and generating the 1-\textit{N}-
oxime metabolite. Thus, we aim to prepare a series of 1,2,4-benzotriazine 1-oxides to find a suitable fluorescent probe, which may display superior brightness compared to the parent metabolite. Compounds 3, and 66-70 were synthesized according to the literature procedures and the 1H NMRs matched literature reports. The 1,2,4-benzotriazine 1-oxide core (3) is typically prepared via the condensation of 2-nitroaniline with cyanamide (Scheme 6.1.2A). Condensation of various 2-nitroaniline derivatives bearing substituents on the benzo ring provides wide varieties of TPZ derivatives. A variety of benzotriazine 1-N-oxide analogues bearing alkyl and aryl substituents on the 3-amino group, compounds 71, 72, 74 and 75 were prepared by nucleophilic aromatic substitution of amine (Scheme 6.1.2B). Synthesis of 5, 6 and 1-N-oxide were prepared by a literature method. Typically, 3-halo-1,2,4-benzotriazine were made from 5 by treatment of sodium nitrite in aqueous sulfuric acid, followed by treatment with phosphorus oxychloride and oxybromide. The halide substrates were coupled with a series of aryl and alkyl boronic acids to yield compound 19, 25-27, 73. (Scheme 6.1.2B). These C-C and N-C coupling method on benzotriazine would certainly give us an opportunity to make a wide verity of TPZ analogues (Scheme 6.1.3). Tirapazamine and di-oxide of 69 were synthesized according to literature method. The mono N-oxide was typically converted to di-N-oxide by a known method using H2O2/AcOH.
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**Scheme 6.2**: Synthesis of various benzotriazine 1-oxide derivatives

**Scheme 6.3**: General strategy of C-C and C-N coupling on BTO

**Scheme 6.4**: The synthetic route of the target molecules
Figure 6.2a: A series of known 1-N-oxides

The extinction coefficients ($\varepsilon$) of the compounds were calculated according to the Beer-Lambert law, and UV spectra of the samples in water (final concentration: 100 $\mu$M) were recorded with a HP Jet direct UV/Vis spectrometer. Solutions of the benzotriazine analogues and standards were measured with absorbance of approximately 0.1–0.5, and were diluted 10 fold for the fluorescence measurement.$^{34}$

Fluorescence spectra were recorded with a RF-5300PC fluorescence spectrophotometer with a range of excitation at 400 nm to 500 nm. The fluorescence quantum yields ($\Phi_F$) were determined by TPZ-mono and fluorescein as references, with a known $\Phi_F$ value of 0.12 in acetonitrile and 0.91 in 0.01 M NaOH, as a reference.$^{35}$ The
area of the emission spectrum was integrated by using instrumentation software, and the quantum yield was calculated according to equation (1), in which $\Phi_F(S)$ and $\Phi_F(R)$ are the fluorescence quantum yields of the sample and the reference, respectively, the terms $A(S)$ and $A(R)$ are the absorbance of the sample and reference solution at the specific excitation wavelength, and $n(S)$ and $n(R)$ are the refractive indices of the solvents used for the sample and reference, where $n(S) = n(R)$.

\[ \Phi_1 = \frac{A_S \Phi_1 (n_S)^2}{A_S \Phi_1 (n_S)^2} \phi_{f,S} \]  

(6.1)

6.3 Results and Discussion of the Structure Fluorescence Activity

A series of derivatives of benzotriazine were synthesized and their fluorescence properties were characterized. Photochemistry of benzotriazine 1-N-oxide and di-oxide is complex. Here, we describe some of the fluorescent properties of heterocycle N-oxides. At first, two functional moieties are essential for these compounds to behave as fluorescent molecules. Replacing exocyclic $3\text{-NH}_2$ of benzotriazine scaffold, significantly diminish the fluorescent property, which depicts the importance of amine group to fluorescence property. In addition, N-O1 moiety is also a required component to display fluorescent property. For example, compounds 5, 73, 6, 19, 25-27, and 43 do not show any significant quantum yields compared to the parent metabolite. Thus, exocyclic amine group and the N–O1 moiety are the essential structural motif in order to maintain its fluorescence property.
Installation of alkyl group on 3-NH2, compounds 71 and 72 show moderate bathochromic shift with reasonable quantum yields, which provides a way for tuning the fluorescence color of this class of benzotriazine derivatives.

<table>
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<th>UV, λmax (nm)</th>
<th>log ε</th>
<th>FI, λmax (nm)</th>
<th>φ</th>
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<tr>
<td>3</td>
<td>405</td>
<td>3.65 (4490)</td>
<td>505 (0.12)</td>
</tr>
<tr>
<td>71</td>
<td>420</td>
<td>3.59 (3936)</td>
<td>510 (0.08)</td>
</tr>
<tr>
<td>72</td>
<td>440</td>
<td>3.59 (3890)</td>
<td>530 (0.06)</td>
</tr>
</tbody>
</table>

**Figure 6.3:** Bathochromic shift from right to left with alkylsubstitution on 3-NH2 in MeCN

Introduction of an aryl group on the 3-amine position, showed no fluorescence property, which can be explained by the quenching of excited state of benzotriazine 1-N-oxide by an aryl group. The fluorescence quantum yield of 68 is increased by installing electron donor substituent on the 7 position of the benzo-ring. Thus, if a weak inductive effect (+I), causes slight positive change in the fluorescence quantum yield and in absorption spectra and emission spectra, then introducing a substituent with mesomeric effect (+M) like methoxy group, might have bathochromic shift and higher quantum yield, compared to the parent metabolite. For example, compound 69 exhibits somewhat higher UV absorption maxima, fluorescence emission maxima and quantum yield (0.46 in acetonitrile). Considering extended planar conjugation might improve the fluorescence,
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either we replaced or substituted the exocyclic 3-aryl type amine with a series of aryl groups, such as compounds 74, 75, and 25–27. All these compounds show very low quantum yields. In addition, 73 shows weak fluorescence with a better extinction coefficient compared to the parent 1-N-oxide (Table 6.1).

To this end, 69 shows longer wavelength absorption, emission maxima, and a significantly improved quantum yield. The increasing quantum yield is due to the methoxy substituent. Introduction of electron donating groups on 8, 6, and 5 position of benzotriazine 1-N-oxide is still under active investigation. With increasing polarity of the solvents (with higher dielectric constant), the fluorescence emission maxima display a systematic red-shift, with no significant change in shape of the spectra (Figure 6.1.5). Compound 69 has moderate quantum yield in water compared to other active benzotriazine 1-N-oxide. Thus, we decided to perform photostability and metabolism studies of 69 under hypoxia.

The fluorometer source is intense enough to photobleach the fluorescent probes, which is considered as one of the drawback for imaging purposes. For example, widely used fluoropore, fluoresein dyes and their conjugates undergo a higher rate of photobleaching process. Interestingly, 69 is highly photostable compared to fluorescein analogs. In this experiment, specific excitation was set for each fluorophore to perform photo-bleaching experiment (Figure 6.1.7). Compound 69 showed a good Stokes shift, and absorption and emission spectra are well separated (100 nm), whereas fluorescein has 25 nm of stoke shift which is low. A larger stoke shift increases sensitivities of fluorescent probe, such as low background interferences and low fluorescence self-quenching effect.
The effect of biologically relevant concentration of thiols on this fluorophore was investigated. We did not notice any significant change of fluorescent intensity, even with the presence of amine, single stranded and double-stranded DNA (Figures 6.2.1, 6.2.2). Thus, this metabolite (6e) could further be improved and, be biologically useful for imaging tumor hypoxia.

We compared the fluorescence spectrum of 7-methoxy-1-oxide with 1-oxide of parent TPZ molecule, and methoxy-1-oxide showed an intense fluorescence emission at about 540 nm upon excitation at a wavelength of 442 to 450 nm. (Figure)

![Figure: Compound 69 and 3 under long UV wavelength](image)

We also compared the fluorescence intensity of 87 and 69, and the fluorescence intensity of 87 was weak. The fluorescence quantum yields (FF) of mono-methoxy and methoxy-tpz were 0.42 and 0.01, respectively. These results indicate that the fluorescence of the methoxy-dioxide is quenched intra molecularly by the N4-oxide moiety. Upon one electron reduction under hypoxia di oxide converted to fluorescent mono-N-oxide. Their molar extinction coefficients were ($\varepsilon = 6000$ and $5000 \text{ M}^{-1}\text{cm}^{-1}$), respectively (Table 6.1.2).

Next, we set out to characterize the biochemical one-electron reduction of methoxy-dioxide, we subjected methoxytpz to enzymatic reduction using X/XO and
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NADPH:cytochrome P450 reductase. Evidence that NADPH:cytochrome P450 reductase is expressed in many pathological tissues stimulated us to carry out the bioreduction of probe. We incubated the probe with NADPH:cytochrome P450 reductase at oxygen concentrations (~1 µM, measured by Oxyprobe). An intense fluorescence emission was observed for the solution of probe incubated with 1 µM oxygen as a model of hypoxia (Figure 6.1.8). At an oxygen concentration of 100 µM, as under aerobic conditions, the extent of enhanced fluorescence intensity diminished significantly. Thus, the probe was activated by NADPH:CypP450 R in a hypoxia-selective manner. We also confirmed the hypoxia-selective metabolism of methoxy di-oxide upon treatment with NADPH:cytochrome P450 reductase, as monitored by HPLC. Next we wanted to study the metabolism of methoxy-tpz with a human prostate cancer cell line (PC-3) and a cultured human colon cancer cell lines HT-29. As a starting point, we worked with the parent compound, TPZ, to optimize the conditions for this cell study. TPZ was incubated at 37 °C for 5 h under hypoxic and aerobic conditions with a human cell lines HT-29 and PC-3. After incubation, the samples were filtered and analyzed by fluorescence spectrophotometry and HPLC. The intensity of the emission was 2 times as strong as that of the sample incubated under aerobic conditions. For PC-3 cell line, TPZ was metabolized under hypoxia to 1-oxide 4 times more than oxic conditions. The lower metabolism can be explained by limited expression of intracellular reductive enzymes such as NADPH/Cyp450 R) from the PC-3 cell lines. These results indicate that methoxyTPZ could undergo one-electron reduction by intracellular reductase to release fluorescent metabolite 1-oxide under hypoxic conditions. Preliminary data showed 2-3 fold more (hypoxia) fluorescent intensity compared to control reaction (data not shown).
These novel studies inspire us to design more 1-N-oxides with methoxy group on the side ring of benzotriazine and an aromatic group forged at 3-positon using our Suzuki-myuara cross coupling method.

The reason we set out to design this mole in future:

i. 7-OMe gives reseaonably good quantum yield, slightly longerwavelenght abs/em

ii. The 3-PhNH2 substituents is hoped to provide longer wavelength abs/em seen in 3-PhOMe derivative, while the free amino residue, albeit attached via a phenyl spacer, was hoped to import some of the brightness (superior quantum yields) typically associated with the 3-amino analogues. This rationally designed analogue will posses superior properties including longer wavelength absorbance, longer wavelength emission, and a reseaonable quantum yield.

6.4 Conclusion and Future Goal

We observed that the antitumor agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (Tpz, 4) and its resulting metabolite exhibited significantly different fluorescence characteristics. Based on the characteristics of the compound 4, which is reduced to 3-amino-1,2,4-benzotriazine 1-oxide under anoxic conditions. Herein, 7-methoxy 3-amino-1,2,4-benzotriazine 1,4-dioxide and its metabolite was designed, synthesized and its photophysical properties were evaluated as a probe. Upon one electron reduction of 4 under hypoxic conditions, significant fluorescence enhancement was measured spectroscopically along with the cell imaging studies.

This benzotriazine scaffold as a hypoxia-selective sensor has not been considered before. 7-OMe-BTO has a moderate fluorescence quantum yield and molar extinction
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Coefficient (brightness = $\Phi \times \varepsilon = 500 \pm 86$. Recently, Tanabe et al. from Japan developed a fluorescent for the detection of hypoxia and worked with a fluorophore having brightness of only 260 ($\Phi \times \varepsilon = 260$).\textsuperscript{36} Typically, the lower limit of practical brightness of a fluorophore is 250 (QY= 0.05 and $\varepsilon = 5000 \text{ M}^{-1}\text{cm}^{-1}$) Tanabe et al. measured the emission intensity of the fluorophore in HT-1080 cell lysate. To conclude, from our detailed study, and understanding on benzotrizine compounds, we envision that our ongoing effort to make brighter fluorophore might have unique properties which favor cancer cells imaging or hypoxia-directed imaging.

6.5 Future Goal 1 In future, we would like to synthesize some fluorescent molecule based of the benzotriazine scaffold as follows:

![Figure 6.4: Proposed fluorescent molecule based on benzotriazine scaffold](image)

These molecules have planar structure and longer conjugation than our recent studied molecules. Longer conjugation will improve the extinction coefficient and methoxy group is considered because it is shown to improves the quantum yield. Considering these two effects, we speculate that we can have a better fluorescent probe in near future.

6.6 Future Goal 2: Hypoxia Sensors based on Fluorescence Resonance Energy Transfer. From our fluorescence study, we have noticed that the emission maxima of the
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1-Noxide fluorescent molecule overlaps with the di-oxide probe. For real life applications, we want the emission maxima of probe and fluorescent in different places in the nano meter scale. To overcome this problem, we propose a fluorescence energy resonance transfer technique.

Proposal and Designing: Choosing right pair for FRET is the crucial step

[Diagram of FRET process]

**Scheme 6.5:** General mechanism of energy transfer in FRET

What should be M1 and M2 in order to get right FRET?

**Category:**

<table>
<thead>
<tr>
<th>Category</th>
<th>Absorption Max (nm)</th>
<th>Emission Max (nm)</th>
<th>Molar Extinction Coefficient</th>
<th>Quantum Yield</th>
</tr>
</thead>
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<td>540-560</td>
<td>~6000.0</td>
<td>0.12</td>
</tr>
<tr>
<td>M2</td>
<td>376</td>
<td>440</td>
<td>0.56 (in CH3CN)</td>
<td></td>
</tr>
</tbody>
</table>

We should have fluorescent molecule, attached to our N-oxide analog which can emit at 440 – 460 nm, and a fluorescent molecule, attached to our N-oxide analog which can absorb at 540- 560 nm range

1. Coumarin derivative

![Coumarin derivative image]  

**Uvmax(abs) = 368 nm, Fl max(em) = 440 nm, mol extic. coeff. = 10500, QY= 0.27**

2. Coumarin derivative

![Coumarin derivative image]  

**Uvmax(abs) = 376 nm, Fl max(em) = 440 nm, mol extic. coeff. = ?, QY= 0.56 (in CH3CN)**
6.7 Proposed Total Synthesis of Benzotriazine Based FRET Molecules

Scheme 6.5: Proposed synthesis of different FRET molecule for hypoxia imaging

A fluorescent molecule which can emit at 440 – 460 nm:

Scheme 6.6: Proposed New FRET probe based on benzotriazine scaffold

Coumarin moiety will absorb at 360 nm and emit at 440 nm. According to FRET theory, the emitted energy at 440 nm may be absorbed by 1-Noxide moiety and light may be
emitted at 540-560 nm as cellular hypoxia sensor. There may not be any energy transfer when the di-N-oxide is present in the molecular building.

6.8 Experimental Section

UV and fluorescence spectra:

<table>
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<tr>
<th>Entry</th>
<th>$\lambda_{\text{max}},$ nm</th>
<th>$\varepsilon$</th>
<th>emission $\lambda_{\text{max}},$ nm</th>
<th>$\phi_f$</th>
<th>brightness ($\varepsilon \times \phi_f$)</th>
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$^a$ Measured in acetonitrile solution, $^b$ For $10^{-7}, 10^{-8}$ M solutions

$^c$ Relative quantum yields from two or three measurements, NM= not significant
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<tr>
<th>entry</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;, nm</th>
<th>ε</th>
<th>emission λ&lt;sub&gt;max&lt;/sub&gt;, nm</th>
<th>φ&lt;sub&gt;f&lt;/sub&gt;</th>
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<sup>a</sup> Measured in water, <sup>b</sup> For 10<sup>-7</sup>, 10<sup>-8</sup> M solutions
<sup>c</sup> Relative quantum yields from two or three measurements, <sup>d</sup> Lit. value, NM= not measured
Chapter 6

Toward Fluorescent Probes of Tumor Hypoxia Based on 1,2,4 Benzotriazine-1-N-oxide Scaffold

<table>
<thead>
<tr>
<th>Compound</th>
<th>UV, λ\text{max} (nm)</th>
<th>(log ε)</th>
<th>FL, λ\text{max} (nm; Φ)</th>
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<tr>
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<td>450</td>
<td>5100</td>
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<tr>
<td>87</td>
<td>480</td>
<td>8000</td>
<td>570, (0.0072)</td>
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</table>

**Table 6.4** Spectra data of compounds 69 and its di-oxide

**Figure 6.5:** UV-vis spectra of 6e and and its dioixde. 100 μM of 3 and 4 in Water was used (5 % DMSO was used as cosolvent, 50 mM NaP pH 7 buffer 69 (green solid line) showed λ\text{max} at 458 nm, ε = 5100 /M/cm, maxima at 442 nm, ε = 5800 /M/cm

**Figure 6.6:** Fluorescence intensities of compounds 69 and dioxide of 87 at the concentration of 10 μM in water. The fluorescence spectra were measured with excitation of 458 and 480 nm. (10 μM compound, 50 mM NaP and 5% DMSO)
6.9 Stokes Shift

**Figure 6.7:** Fluorescence spectra of 3 (10 μM) in water. From left to right: Excitation spectrum, $\lambda_{em} = 550$ nm; Emission spectrum, $\lambda_{ex} = 450$ nm. Stokes shift was measured by taking the difference between $\lambda_{em}$ and $\lambda_{ex}$. Fluorescein has stokes shift of about 20 nm.

6.10 Photostability Experiment

**Figure 6.8:** Photostability experiments of OMe-BTO (0.1 μM, 50 mM NaP pH 7 buffer) and Fluorescein (0.1 μM, 50 mM NaP pH 7 buffer). Excitation wavelength at 458 nm and 490 nm were used respectively. Emission intensities were collected at 550 nm and 510 nm.
6.11 Metabolism Studies and Fluorescence Output:

The fluorescence spectra were measured with excitation of 458 and 480 nm. Bold line represents intensity of enzymatic reaction of 7-methoxy probe under anaerobic conditions and dashed line represents a control reaction with no enzyme under similar conditions.

**Figure 6.9:** Fluorescence responses of methoxy probe under anaerobic conditions after treatment with reductase. 100 μM of 7-methoxy probe was incubated with sodium phosphate buffer (50 mM, pH 7.0), xanthine (500 μM) and xanthine oxidase (0.4 mU/mL) reductase under anaerobic conditions at room temperature for 4 h.
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Figure 6.91  Fluorescence responses of 87 under aerobic and anaerobic conditions after treatment with reductase. 100 μM of 7-methoxy probe was incubated with sodium phosphate buffer (50 mM, pH 7.0), xanthine (500 μM) and xanthine oxidase (0.4 mU/mL) reductase under aerobic and anaerobic conditions at room temperature for 4 h. The fluorescence spectra were measured with excitation of 458 and 480 nm. Bar 1, compound 4 + O₂ (no enzyme); Bar 2, compound 4 + reductase 0.4U/mL + O₂; Bar 3, compound 4 (no enzyme); Bar 4, compound 4 + reductase 0.4U/mL (under anaerobic conditions)

Figure 6.92. Photograph of solutions of 69 and its probe (100 μM in 5% DMSO in water) irradiated with a UV lamp. A, probe 87; B, 69
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6.12 Metabolism Study by TLC and MS method: Redox Activated in vitro

Metabolism of 7-methoxy 1,2,4-benzotriazine 1,4-dioxide under Hypoxic Conditions

Yields the 1-N-Oxide as the Major Metabolite

Our group and others have extensively studied on the metabolism of TPZ and various TPZ analogs.\textsuperscript{16,17,18} In presence of NADPH:cytochrome P450 reductase or xanthine/xanthine oxidase, TPZ yields the 3-amino-1,2,4-benzotriazine-1-oxide as the major metabolite. Identification of metabolites is an important part of the in vitro biochemical studies to understand the mechanism of DNA strand cleavage by TPZ, and by other various heterocyclic N-oxides. We examined the products generated by one-electron reductive activation of this compound by the enzyme NADPH:cytochrome P450 reductase under anaerobic conditions. The reaction mixture was extracted three times with ethyl acetate (200 µL each time), collected and air dried. Then sample was spotted on TLC plates. All the TLC plates were run at 40% ethyl acetate in hexanes. Then the silica gel was scraped off and redissolved in ethyl acetate and filtered through a regular column. It was dried and collected and compared with the authentic standard sample. Thin layer chromatography (TLC) followed by mass spectrometry.

6.13 Measurement of Fluorescence Quantum Yield

The fluorescence quantum yield ($\Phi_F$) was determined by fluorescein, with a known $\Phi_F$ value of 0.91 in water (0.01 M NaOH), as a reference. The area of the emission spectrum was integrated by using instrumentation software, and the quantum yield was calculated according to equation (1), in which $\Phi_F(S)$ and $\Phi_F(R)$ are the fluorescence quantum yields of the sample and the reference, respectively, the terms $A(S)$
and $A_{(R)}$ are the optical densities of the sample and reference solution at the excitation wavelength, and $n_{(s)}$ and $n_{(R)}$ are the refractive indices of the solvents used for the sample and reference.

### 6.14 Fluorescent Intensity Ratio of 7-methoxy 1,2,4-benzotrizine 1-Oxide and its di-N-oxide

7-OMe-1-oxide and its di-oxide were mixed in the following proportion to observe the fluorescence intensity output.

**Excitation wavelength 458 nm**

<table>
<thead>
<tr>
<th></th>
<th>yield of mono</th>
<th>(integrated area of mono in the mixture)/di</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10 uM di-oxide</td>
<td>0</td>
<td>0/3988</td>
</tr>
<tr>
<td>2. 9 : 1 di : mono</td>
<td>10%</td>
<td>9100/3988 = ~ 2X</td>
</tr>
<tr>
<td>3. 8 : 2 di : mono</td>
<td>20%</td>
<td>12000/3988 = ~ 3X</td>
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<tr>
<td>4. 7 : 3 di : mono</td>
<td>30%</td>
<td>16100/3988 = ~ 4X</td>
</tr>
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</table>

1. 9 uM of OM-tpz vs 1 uM of OM-tpz-mono (excitation was used at 458 nm)

2. 8 uM of OM-tpz vs 2 uM of OM-tpz-mono

Area of 1 uM mono / area of 9 uM OM-tpz = 8909 / 4332 = 2X

Area of 2 uM mono / area of 8 uM OM-tpz = 11750 / 4049 = 3X
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![Figure 6.93](image)

**Figure 6.93**: Relative Fluorescence Intensity Comparisons of 7-methoxy 1-oxide and di-oxide

### 6.15 *In vitro* Metabolism Studies

OMe-Tpz was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 1% or less. PC-3 and HT-29 colon cancer cells were used for this study. OMe-Tpz was added to cell suspensions to give 50 µM drug concentration. The cell and drug suspension was incubated in a chamber with gases (air+5%CO2, nitrogen+5% CO2) at 37 °C. Concentration of the media was measured by using Oxygen-probe. After 5 hours of incubation, cell samples were centrifuged and sonicated to crack open the cell wall. Then the cell suspensions were evaluated by Fluorescence spectroscopy and using appropriate excitation wavelength. Cell suspensions were also extracted with organic solvent such as dichloromethane and ethyl acetate and then analyzed by fluorescence spectroscopy.
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<table>
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<th>TPZ (peak area)</th>
<th>Mono (peak area)</th>
<th>Ratio</th>
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<td>401</td>
<td>159</td>
<td>0.39</td>
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<tr>
<td>2. Anaerobic lane + tpz</td>
<td>447</td>
<td>261</td>
<td>0.59</td>
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<tr>
<td>3. media + tpz</td>
<td>663</td>
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<tr>
<td>5. 50 uM mono tpz</td>
<td>720</td>
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50 uM shows peak area of 2434
16.6 uM should show area of 808

Preliminary data (Without cracking the cells)
- The instrumental setup for the fluorescence studies --- Ex = 415 (for mono-N-oxide fishing) 10 + 10 PL

<table>
<thead>
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<th>Area</th>
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<tr>
<td><strong>Eppy 3. TPZ (50 uM) + Cell + O₂ (21%)</strong></td>
<td>30432</td>
</tr>
<tr>
<td><strong>Eppy 2. TPZ (50 uM) + Cell + O₂ (0.1%)</strong></td>
<td>35118</td>
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release of ~1-2 uM mono-N-oxide
cross checked with 1 and 5 uM of Tpz-mono (freshly prepared solution in water) and then the yield was calculated.
6.16 Fluorescence Quenching Studies With Thiol and Amine
Figure 6.95  Fluorescence quenching experiments of OMe-BTO (69), A; and OMe-Di-oxide (87). OMe-BTO (5 μM, 50 mM NaP pH 7 buffer, 10 mM of beta-mercaptoethanol and 10 mM of diethylamine). Excitation wavelength at 458 nm and 480 nm were used respectively. Emission intensities were collected at 550 nm.
References


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73. Jiang, Faqin; Yang, Bo; Fan, Lingling; He, Qiaojun; Hu, Yongzhou Bioorg. Med. Chem. Lett. 2006, 16, 4209-4213


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Ujjal Sarkar, the youngest son among three children was born on January 11, 1979 in Jagacha, Howrah, India, the youngest son of Umesh C. Sarkar and Manoroma Sarkar. He did his secondary (tenth grade) education from Jagacha High School, West Bengal in 1995. After completing his higher secondary (twelfth grade) education at Howrah Vivekananda Institution in 1997, he entered Presidency College, Calcutta, and received the degree of Bachelor of Science in Chemistry with honors in 2000. He entered Indian Institute of Technology, Kharagpore, and received a Master's degree in Chemistry in 2002. After completing his Master degree, he joined University of Missouri to pursue his PhD in Chemistry. He decided to work with Dr. Kent S. Gates, and began working in his group from January 2004. During his stay at University of Missouri, he did excellent teaching services as a teaching assistant, and was recognized by Breckenridge/Lyons Outstanding Graduate Teaching Award in 2008 from the department of chemistry. Ujjal was married to Mita Das in the winter of 2007 in Kalyani, India. Mita is currently doing her PhD in Mathematics at Mizzou. They were blessed with a wonderful child Umasrija Sarkar in September of 2009. Ujjal graduated in the fall of 2009, and is currently working with Dr. Steven R. Tannenbaum in the Biological Engineering Department at MIT, as a postdoctoral fellow. Besides chemistry, he acted as editors and associate editors for several online magazines for art, literature and science. Ujjal is also an active member of
Cultural Association of India and The Vedic Society, recognized student organization at Mizzou. He likes to play Indian drums and harmonica, loves to paint and enjoys creative cooking as well.