Chemical Research in Toxicology

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Isotopic Labeling Experiments That Elucidate the Mechanism of DNA Strand Cleavage by the Hypoxia-Selective Antitumor Agent 1,2,4-Benzotriazine 1,4-Di-*N*-oxide

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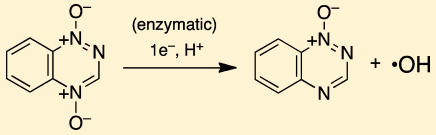
Supporting Information

ABSTRACT: The 1,2,4-benzotriazine 1,4-dioxides are an important class of potential anticancer drugs that selectively kill the low-oxygen (hypoxic) cells found in solid tumors. These compounds undergo intracellular one-electron enzymatic reduction to yield an oxygen-sensitive drug radical intermediate that partitions forward, under hypoxic conditions, to generate a highly reactive secondary radical that causes cell killing DNA damage. Here, we characterized bioreductively activated, hypoxia-selective DNA-strand cleavage by 1,2,4-benzotriazine 1,4-dioxide. We found that one-electron enzymatic activation of 1,2,4-benzotriazine 1,4-dioxide under hypoxic conditions in the presence of the deuterium atom donor methanol-d_4 produced nondeuterated mono-*N*-oxide metabolites. This and the results of other isotopic labeling studies provided evidence against the generation of atom-abstracting drug radical intermediates and are consistent with a DNA-damage mechanism involving the release of hydroxyl radical from enzymatically activated 1,2,4-benzotriazine 1,4-dioxides.

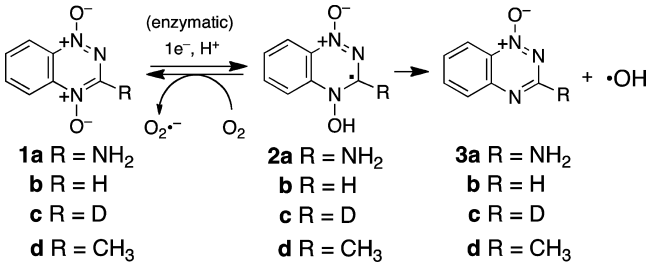


INTRODUCTION

In cancer chemotherapy, there is a continuing need for the development of drugs that selectively target malignant tissue.[1] Toward this end, the poorly developed vascular system in tumors creates a low oxygen environment that may be exploited by some small molecules for the selective killing of cancer cells.[2] The 1,2,4-benzotriazine 1,4-di-*N*-oxides are an important class of hypoxia-selective cytotoxins.[2−7] Tirapazamine (**1a**) is the lead compound in this class of drugs, but many analogues in this structural family display similar bioactivities,[3−6] and the relatively new agent SN30000/CEN-209 (**4**) is advancing toward clinical development.[8,9]

The medicinal action of tirapazamine and other 1,2,4-benzotriazine 1,4-dioxides involves intracellular one-electron enzymatic reduction to yield an oxygen-sensitive drug radical intermediate (**2**).[10−13] In normally oxygenated tissue, **2** is simply oxidized by O_2 back to the parent compound **1**.[13,14] However, under hypoxic conditions, the radical intermediate **2** partitions forward to generate a highly reactive secondary radical that kills cells via abstraction of hydrogen atoms from the 2′-deoxyribose phosphate backbone of DNA.[15−22] The mono-*N*-oxide **3** is the major drug metabolite generated in this process.[15,16,23] The exact nature of the ultimate DNA-damaging radical generated following bioreductive activation of the 1,2,4-benzotriazine 1,4-dioxides remains a topic of ongoing study, with two different possibilities considered in the recent literature. First, evidence has been presented that is consistent with the release of hydroxyl radical (HO·) from the protonated drug radical **2** (Scheme 1).[21,24−28] This mechanism adequately

Scheme 1



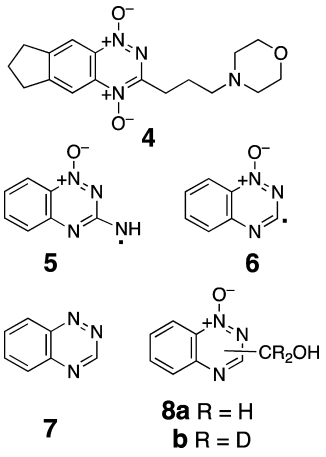
accounts for the DNA-damaging properties and the hypoxia-selective cytotoxicity of many different heterocyclic *N*-oxides including 1,2,4-benzotriazine 1,4-dioxides,[21,24−26,29] quinoxaline 1,4-dioxides,[30−33] 1,2-dihydro-4-phenylimidazo[1,2-a]pyrido-[3,2-e]pyrazine 5-oxides,[34] and phenazine 5,10-dioxides.[35] A second mechanistic proposal suggests that the tirapazamine radical **2** can undergo dehydration to generate a highly reactive benzotriazinyl radical **5** that abstracts hydrogen atoms from DNA.[36−40] To account for the bioactivities of other 1,2,4-benzotriazine 1,4-dioxide analogues such as **1b**, the dehydration mechanism was expanded to include a family of hypothetical processes that generate aryl radical intermediates such as **6**.[38]

Isotopic labeling experiments have the potential to distinguish which of these two mechanistic pathways is

Received: September 27, 2013
Published: December 11, 2013

dx.doi.org/10.1021/tx400356y | *Chem. Res. Toxicol.* 2014, 27, 111−118

operative. For example, the proposed generation of the aryl radical **6** from **2b** in the presence of a deuterium atom donor would yield the corresponding deuterated drug metabolite **3c**,[41,42] whereas release of the hydroxyl radical from **2b** will produce the nondeuterated metabolite **3b**. Here, we report the results of such isotopic labeling studies carried out in the context of compound **1b**. We reasoned that **1b** was a good subject for mechanistic studies because this agent displays hypoxia-selective cytotoxicity comparable to that of tirapazamine (**1a**)[3] and has been proposed to generate the DNA-damaging aryl radical **6**.[38] However, the ability of **1b** to carry out in vitro reductively activated, hypoxia-selective DNA strand cleavage in a manner similar to that of tirapazamine had not been previously demonstrated. Therefore, before undertaking isotopic labeling studies, we characterized the DNA-damaging properties of **1b**.[13,21,25,43] We found that **1b** displays bioreductively activated, hypoxia-selective DNA-cleaving properties analogous to those of the lead compound tirapazamine (**1a**). The results of several different isotopic labeling studies provided evidence against the generation of atom-abstracting drug radicals such as **6** following one-electron enzymatic activation of **1b** under hypoxic conditions.



MATERIALS AND METHODS

Materials. Materials were of the highest purity available and were obtained from the following sources: Sephadex G-25, cytochrome P450 reductase, NADPH, sodium phosphate, mannitol, DMSO, desferal, catalase, superoxide dismutase (SOD), *tert*-butyl nitrite, silica gel (0.04−0.063 mm pore size) for column chromatography, and silica gel plates for thin layer chromatography from Sigma Chemical Co. (St. Louis, MO); 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); and deuterated NMR solvents (deuterium oxide, D 99.96%, methanol-d_4, D 99.8%, *N*,*N*-dimethylformamide-d_7, D 99.5%; and acetone-d_6, D 99.8%) were from Cambridge Isotope Laboratories (Andover, MA). Compounds **1a** and **1b** were prepared by routes described previously in the literature.[3,23,44] Compound **3b** was prepared via the same general procedure used for **1b**,[3] and the spectral data of this product matched that published previously.[38]

DNA Cleavage Assays. DNA strand cleavage reactions were carried out as described previously.[32,35] Briefly, in a hypoxic DNA-cleavage assay, agents such as **1a**, **1b**, or **1d** (25−150 μM) were incubated with supercoiled plasmid DNA (33 μg/mL, pGL-2 Basic), NADPH (500 μM), cytochrome P450 reductase (33 mU/mL), where one unit is defined as the amount of enzyme required to cause the reduction of 1.0 μmole of cytochrome *c* by NADPH per minute at pH 7.4 at 37 °C), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0), acetonitrile (0.5−3.3% v/v), and desferal (deferoxamine mesylate salt, 10 mM) under anaerobic conditions at 25 °C for 4 h. All components of the reactions except enzymes, NADPH, and DNA were degassed by three freeze−pump−thaw cycles. Enzymes, NADPH, and DNA were diluted with degassed water in an argon-filled glovebag to prepare stock solutions. Reactions were initiated by the addition of cytochrome P450 reductase, wrapped in aluminum foil to prevent exposure to light, and incubated in an argon-filled glovebag. Following incubation, the reactions were quenched by the addition of 4 μL of loading buffer containing bromophenol blue dye and were loaded onto a 0.9% agarose gel. The gel was electrophoresed for approximately 2 h at 83 V in 1 × TAE buffer. The gel was then stained in a solution of aqueous ethidium bromide (0.5 μg/mL) for 15 min, destained in fresh distilled water for 15 min, and the DNA in the gel visualized by UV-transillumination. The amount of DNA in each band was quantified using an Alpha Innotech IS-1000 digital imaging system. Buffers for the reactions performed in D_2O were prepared using the same mixture of phosphate buffer salts used in the H_2O experiments, as described by others.[45,46]

Synthesis of 4-Deutero-1,2,4-benzotriazine 1,4-Dioxide (1c). In a 25 mL round-bottomed flask, *tert*-butyl nitrite (0.25 mL, 2.10 mmol) was added to DMF-d_7 (2 mL) and heated to 65 °C. To this mixture, a solution of **1a** (100 mg) in DMF-d_7 (2 mL) was added dropwise over the course of 15 min. The reaction was monitored by thin layer chromatography every 10 min until all starting material was consumed. The reaction mixture was then cooled to room temperature, and the DMF-d_7 was removed by high vacuum. Column chromatography on silica gel eluted with 1:1 ethyl acetate−hexane gave **1c** as a yellow solid (23 mg, 25%). R_f 0.60 (100% ethyl acetate). ^{1}H NMR (DMSO, 500 MHz,): δ 8.39 (d, J = 8.5 Hz, 1H), 8.36 (d, J = 8.5 Hz, 1H), 8.14 (ddd, J = 8.5, 7.5, 1.0 Hz, 1H), 8.10 (ddd, J = 8.5, 7.5, 1.0 Hz, 1H). ^{13}C NMR (DMSO, 125.8 MHz): δ 142.2 (t, J = 33.3 Hz), 141.0, 136.0, 135.6, 133.3, 121.5, 119.4. HRMS (ES^+, [M + H]) m/z calcd $C_7H_5DN_3O_2$ calcd mass, 165.0523; actual mass, 165.0515.

Synthesis of 1,2,4-Benzotriazine (7). Benzotriazine 7 was prepared via a modification of the procedures described by Fuchs et al. and Mason and Tennant.[23,47] To a solution of **3b** (300 mg, 2.04 mmol) in 70% ethanol−water (40 mL) was added sodium dithionite (710 mg, 4.08 mmol). The resulting suspension was refluxed for 2 h, an additional aliquot of sodium dithionite (305 mg, 2.04 mmol) added, and the suspension refluxed for another 30 min, at which time all starting material was consumed (as judged by TLC). The solvent was removed by rotary evaporation and the resulting pale yellow residue purified by column chromatography on silica gel eluted with 30% ethyl acetate−hexane to yield 7 as a pale yellow solid (154 mg, 58%). R_f = 0.57 (30% ethyl acetate/hexane). ^{1}H NMR (DMSO, 500 MHz,): δ 10.13 (s, 1H), 8.61 (d, J = 8.5 Hz, 1H), 8.18−8.24 (m, 2H), 8.10 (ddd, J = 8.5, 6.5, 1.5 Hz, 1H). ^{13}C NMR (DMSO, 125.8 MHz): δ 154.1, 148.1, 140.1, 136.8, 132.2, 129.5, 129.0. Spectral data matched that reported by Boyd et al.[48]

LC-MS/MS Analysis of Mixtures Generated by in Vitro Hypoxic Metabolism of 1b and 1c. In the LC-MS isotope incorporation assay, all stock solutions were made in D_2O except for **1b**. Stock solutions used in the assays were prepared as follows: a solution of **1b** (15 mM) was prepared by dissolving 10 mg of **1b** in 4 mL of acetonitrile, a solution of sodium phosphate (500 mM) was prepared from 409 mg of Na_2HPO_4 and 254 mg of NaH_2PO_4 dissolved in 10 mL of D_2O, a solution of desferal (10 mM) was prepared by dissolving 3 mg of desferal in 455 μL of D_2O), a solution of NADPH (30 mM) was prepared by dissolving 1 mg of NADPH sodium salt in 40 μL of D_2O, the stock of cytochrome P450 reductase (120 U/mL) was prepared by dissolving 3 μL of a 260.4 U/mL stock solution of enzyme dissolved in 3.5 μL of D_2O, and a solution of CD_3OD (15 M) was prepared from 610 μL of CD_3OD in 390 μL D_2O. All stock solutions except cytochrome P450 reductase were degassed by three cycles of freeze−pump−thaw on a vacuum line. For preparation of the assays, the dioxide **1b** (10 μL) was mixed with desferal (30 μL), sodium phosphate buffer (30 μL), NADPH (10 μL), CD_3OD (10 μL of the 15 M CD_3OD stock in D_2O), and 209 μL of D_2O. Finally, the reactions were inititated by the addition of

cytochrome P450 reductase stock solution (1 μL). The final volume of the assay mixture was 300 μL. These assays contained small amounts of residual exchangeable protium. The final mixtures contained approximately 99.5% D content in the water, and thus, small amounts (~0.5%) of CD_3OH will be present alongside much larger amounts of CD_3OD. Nonetheless, these traces of protium in the deuterium atom donor are not expected to represent a confounding factor in the interpretation isotope incorporation assays. Bond enthalpies significantly favor atom abstraction from the methyl group of methanol rather than the hydroxyl group (see Supporting Information for further discussion of this topic). Experimental evidence presented below confirmed that atom abstraction in this system occurs at the methyl group of methanol. Specifically, the formation of significant amounts of metabolites **8** clearly indicated that $\cdot CD_2OH/D$ and $\cdot CH_2OH$ were generated. In addition, assays in which additional steps were taken to remove the residual exchangeable protium sources gave identical results (Figure S8, Supporting Information).

After 4 h of incubation under argon at 25 °C, the proteins in the assay mixtures were removed by centrifugation through Amicon Microcon (YM3) filters. The filtrate was analyzed by Beckman Coulter HPLC employing a C18 reverse phase Betabasic column (5 μm particle size, 150 Å pore size, 25 cm length, and 4.6 mm i.d.) eluted with gradient starting from 5% B (0.1% trifluoacetic acid in acetonitrile) and 95% A (0.1% trifluoacetic acid in water) for 5 min followed by linear increase to 11% B in another 24 min, then the gradient was increased linearly to 80% of B within in 6 min, and finally 80% of B was used to wash the column for 10 min. A flow rate of 1.0 mL/min was used, and the products were monitored by their UV-absorbance at 240 nm. LC/ESI-MS experiments were carried out using an ion trap mass analyzer, on a LCQ FLEET instrument (Thermo Fisher Scientific). Positive ion electrospray was used as the means of ionization. The heated inlet capillary temperature was 375 °C, and electrospray needle voltage was 5 kV. Nitrogen sheath gas was supplied at 45 psi, and the LC/ESI-MS analysis was done in the positive ion mode. Relative collision energies of 35% were used when the ion trap mass spectrometry was operated in the MS/MS mode. Parent ions were selected manually (164, 165, 148, 149, 132, 133, 178, and 180), and subsequent tandem mass spectrometry was performed automatically by XCalibur software (Thermo Fisher Scientific).

RESULTS AND DISCUSSION

Bioreductively-Activated, Hypoxia-Selective DNA Strand Cleavage by 1b. We synthesized **1b** by a literature route involving treatment of **1a** with *t*-butyl nitrite in DMF.[3,44] We prepared the expected metabolite **3b** via the analogous reaction on **3a**.[38] In addition, we prepared a second expected[15,16,23] metabolite **7** by treatment of **3b** with sodium dithionite in ethanol–water. We employed a plasmid-based assay to measure the ability of **1b** to cause bioreductively activated DNA strand cleavage under hypoxic conditions. In this assay, oxidative DNA strand cleavage causes conversion of a supercoiled plasmid substrate (form I) to the open circular form II.[49−52] These two forms of plasmid DNA are then separated using agarose gel electrophoresis and visualized by staining with ethidium bromide.[49−52] This assay is especially useful for monitoring the direct DNA strand breaks generated by radical intermediates.[53,54] Direct strand breaks induced by radicals typically arise via abstraction of hydrogen atoms from the 2-deoxyribose phosphate backbone of DNA.[53,54] We used recombinant human NADPH:cytochrome P450 reductase to carry out one-electron reductive activation of **1b** because this or a related enzyme[55] is thought to be responsible for the intracellular activation of 1,2,4-benzotriazine 1,4-dioxides.[43,56,57] For reactions carried out under hypoxic conditions, molecular oxygen was removed from stock solutions by freeze–pump–thaw degassing and the assay mixtures assembled and incubated in an inert atmosphere glovebag. Catalase, superoxide dismutase, and desferal were included as described previously[21,32] to prevent potential background strand cleavage stemming from the conversion of adventitious molecular oxygen to reactive oxygen species.

We found that **1b** caused DNA strand cleavage when incubated with the plasmid substrate and the NADPH/NADPH:cytochrome P450 reductase enzyme system under hypoxic conditions (Figure 1). Across a range of concentrations

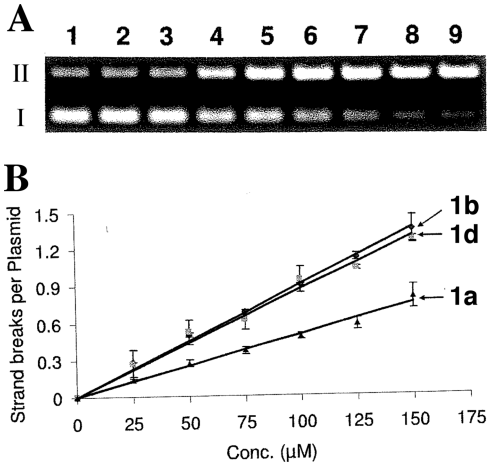


Figure 1. Bioreductively activated DNA strand cleavage by **1b** under hypoxic conditions. (Panel A) Concentration-dependent DNA strand cleavage by **1b**. Supercoiled plasmid DNA (pGL2-basic, 33 μg/mL) was incubated with **1b** (25−150 μM), NADPH:cytochrome P450 reductase (33 mU/mL), NADPH (500 μM), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM) under anaerobic conditions at room temperature for 4 h, followed by agarose gel electrophoretic analysis. Strand breaks per plasmid DNA molecule (S) were calculated using the equation $S = -\ln f_I$, where f_I is the fraction of plasmid present as form I. Lane 1, DNA alone ($S = 0.35 \pm 0.14$); lane 2, enzyme system alone ($S = 0.32 \pm 0.11$); lane 3, **1b** alone (150 μM, $S = 0.38 \pm 0.17$); lanes 4−9, enzyme system + **1b** at 25 μM ($S = 0.49 \pm 0.13$), 50 μM ($S = 0.76 \pm 0.05$), 75 μM ($S = 1.07 \pm 0.08$), 100 μM ($S = 1.30 \pm 0.09$), 125 μM ($S = 1.42 \pm 0.07$), and 150 μM ($S = 1.62 \pm 0.14$). (Panel B) Comparison of concentration-dependent DNA strand breakage by **1a**, **1b**, and **1d** under hypoxic conditions. Strand cleavage by various concentrations (25−150 μM) of the three different compounds was carried out as described above. Background cleavage present in the untreated plasmid was subtracted to allow direct comparison of DNA cleavage yields between different experiments.

(25−150 μM), the yields of DNA strand breaks generated by **1b** were comparable to those produced by **1a** and **1d**, two biologically active compounds whose DNA-strand cleaving properties have been characterized previously (Figure 1).[3,13,21,25,43] Control experiments showed that neither **1b** alone nor the NADPH/NADPH:cytochrome P450 reductase enzyme system (without **1b**) caused significant amounts of DNA strand cleavage (Figure 1). Likewise, when **1b** was incubated with the enzyme alone (no NADPH), NADPH alone (no enzyme), or with the complete enzymatic reducing system under aerobic conditions, no significant amounts DNA strand cleavage were observed (Figure S1, Supporting Information). Further control experiments showed that compounds **3b** and **7**, two major metabolites generated in the hypoxic metabolism of **1b** (see below), did not cause DNA strand cleavage either alone or in the presence of the NADPH:cytochrome P450 reductase enzyme system under hypoxic conditions. We found that DNA

dx.doi.org/10.1021/tx400356y | Chem. Res. Toxicol. 2014, 27, 111−118

strand cleavage by **1b** was substantially inhibited (60−90%) by the classical radical-scavenging agents[58] ethanol, methanol, *t*-butanol, DMSO, and mannitol (500 mM, Figure 2), analogous

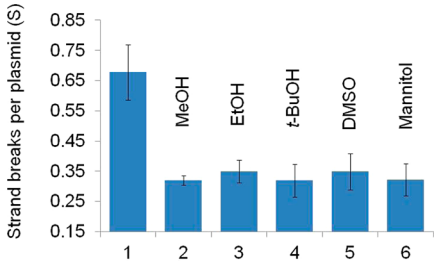


Figure 2. Radical-scavenging agents inhibit bioreductively activated DNA strand cleavage by **1b**. Column 1 shows the strand cleavage yield generated by **1b** (50 μM) under the standard hypoxic reaction conditions described in the Materials and Methods and the legend to Figure 1. Columns 2−6 illustrate the cleavage yields under those same standard conditions except in the presence of the indicated radical scavenging agent (500 mM). Background cleavage present in the untreated plasmid was subtracted to allow direct comparison of DNA cleavage yields between different experiments. The number of strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$, where f_1 is the fraction of plasmid present as form I.

to the results observed previously for **1a** and **1d**. Of special importance to the studies described below, we found that methanol-d_4 (500 mM) efficiently quenched the DNA-cleaving intermediate generated upon enzymatic activation of **1b** (Figure S2, Supporting Information). Overall, the reductively activated, hypoxia-selective DNA-cleaving properties of **1b** closely resemble those of tirapazamine and other 1,2,4-benzotriazine 1,4-dioxides.[13,21,25,43]

In Vitro Bioreductive Metabolism of 1b. In the case of other 1,2,4-benzotriazine 1,4-dioxides that have been characterized, DNA strand cleavage is accompanied by the generation of the corresponding 1,2,4-benzotriazine 1-oxide **3** as the major metabolite (Scheme 1).[15,23,25,27,28] Here, we used LC-MS/MS analysis to characterize the products generated by in vitro, cell-free, hypoxic metabolism of **1b** by NADPH:cytochrome P450 reductase in the presence of methanol as a hydrogen atom source. UV−vis detection of the resulting products (240 nm) revealed a mixture consisting of the starting material **1b** alongside several new metabolites (Figure 3). MS/MS analysis of the products, along with comparison to authentic synthetic standards, showed that the 1,4-dioxide **1b** eluted at 6.5 min, the expected mono-*N*-oxide **3b** eluted at 18.8 min, and that the expected "no-oxide" metabolite **7** eluted at 17.7 min (Table 1).

Table 1. LC-MS/MS Properties of the Mixture Generated by in Vitro Metabolism of 1b under Hypoxic Conditions in the Presence of CH_3OH

metabolites	retention time	$[M + H]^+$	MS/MS fragments
1b	6.5	164	147, 137, 119, 93, 92, 65
7	17.7	132	105, 77
3b	18.8	148	120, 93, 92, 65
8a	14.6	178	160, 132, 120, 104, 93, 92, 77, 65

The minor metabolite eluting at 14.6 min was tentatively assigned the structure **8a** based upon its m/z of 178 in the LC-MS. Such a product could arise by a number of different mechanisms including addition of the methanol-derived radical, $\cdot CH_2OH$, to **1b**, followed by either dehydration or loss of $HO\cdot$.[59] An analogous mono-*N*-oxide product was previously observed to arise via the reaction of the methyl radical with **1a**.[59]

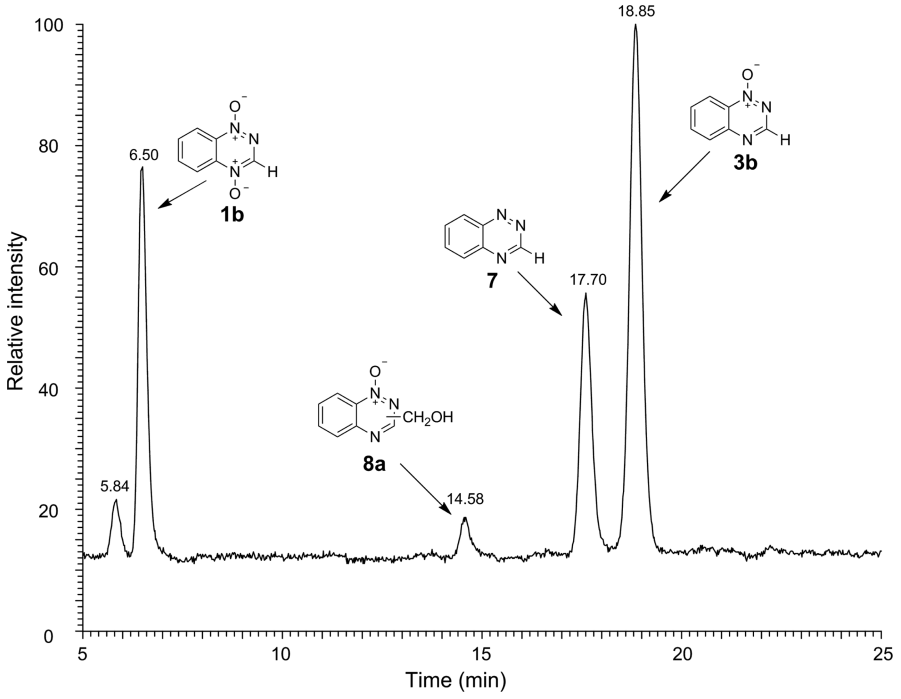


Figure 3. Reverse-phase HPLC chromatogram (UV 254 nm) of the products generated by in vitro hypoxic metabolism of **1b** in the presence of methanol.

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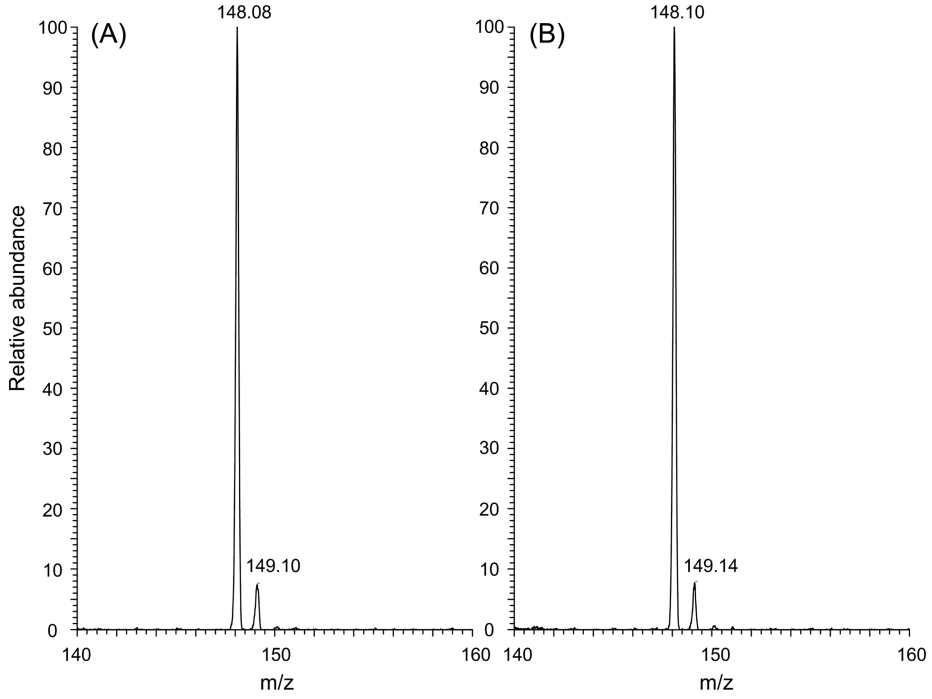

Figure 4. Hypoxic metabolism of **1b** in phosphate-buffered D_2O/CD_3OD does not lead to incorporation of deuterium into metabolite **3**. LC-MS of the mono-*N*-oxide metabolite **3** generated by hypoxic metabolism of **1b**. (Panel A) Isotope cluster for the $[M + H]^+$ ion of **3** generated in phosphate-buffered H_2O/CH_3OH (relative peak intensities for m/z 148:149:150 = 100:7.9:0.5). (Panel B) Isotope cluster for the $[M + H]^+$ ion of **3** generated in phosphate-buffered D_2O/CD_3OD (relative peak intensities for m/z 148:149:150 = 100:8.2:0.6).

In Vitro Bioreductive Metabolism of 1b in Phosphate-Buffered D_2O-methanol-d_4. The results described above set the stage for isotopic labeling experiments designed to probe whether bioreductive activation of **1b** generates an atom-abstracting drug radical intermediate such as **6**. As noted above, the generation of an aryl radical such as **6** from **2b** in the presence of a deuterium atom donor will yield the corresponding deuterated drug metabolite **3c**,[41,42] whereas the release of the hydroxyl radical from **2b** will generate the nondeuterated metabolite **3b**. It may be important to reiterate that the experiments described above demonstrated that methanol-d_4 efficiently quenches bioreductively activated DNA strand cleavage by **1b**, thus providing a clear indication that the DNA-cleaving intermediate generated following bioreductive activation of **1b** does indeed react with methanol-d_4.

Thus, **1b** was incubated with the NADPH:cytochrome P450 reductase enzyme system under hypoxic conditions in phosphate-buffered D_2O containing methanol-d_4 (500 mM), and the metabolites generated in this reaction were analyzed by LC-MS/MS. We observed that the spectrum of metabolites generated in the presence of phosphate-buffered D_2O/methanol-d_4 was identical to that observed in the H_2O/methanol experiment shown in Figure 3 (Figure S3, Supporting Information). Importantly, LC-MS analysis revealed no significant deuterium incorporation into the major metabolite **3** (Figure 4). The relative abundances of ions in the isotope cluster for the $[M + H]^+$ ion of **3** observed in these experiments matched that expected to arise from the natural abundances of ^2H and ^{13}C in a molecule of this molecular formula. Similarly, the no-oxide metabolite showed no deuterium incorporation. Our LC-MS analysis revealed that the mass-to-charge ratio of the metabolite eluting at 14.7 min generated in the D_2O/methanol-d_4 experiment was two Daltons greater than the analogous product generated in the presence of nondeuterated methanol (Figure S4, Supporting Information). This observation is consistent with the notion that the 14.7 min metabolite contains a CH_2OH or CD_2OH fragment derived from methanol or methanol-d_4 (the deuterium on the hydroxyl group of the CD_2OD fragment presumably was exchanged with protons from trifluoroacetic acid in the HPLC eluent during the LC-MS analysis). As noted above, **8a** and **8b** could reasonably arise via multiple reaction pathways; therefore, we cannot consider the formation of these products diagnostic for any particular mechanism.

Bioreductive Metabolism of the Deuterium-Containing Analogue 1c. Finally, we synthesized the deuterated analogue **1c** via treatment of **1a** with *t*-butyl nitrite in DMF-d_7.[44,60] Availability of this labeled compound enabled us to undertake isotope-tracking experiments complementary to the solvent-labeling experiments described above. Specifically, the dehydration mechanism proposed[38] to generate radical **6** would result in the *loss* of deuterium content during the conversion of **1c** to **3** when the reaction is carried out in the presence of protic solvents and hydrogen atom sources. In contrast, the hydroxyl radical mechanism will proceed with complete retention of the label in the conversion of **1c** to **3**. LC-MS analysis of the mixture generated by NADPH:cytochrome P450 reductase-catalyzed metabolism of the deuterated analogue **1c** revealed no significant loss of deuterium content in the enzymatic transformation of **1c** to the corresponding mono-*N*-oxide metabolite **3** (Figure S5, Supporting Information). The observation that the deuterium of the starting material **1c** was completely retained in **3** had the side benefit of demonstrating that a deuterium label does not "wash out" of metabolite **3** under our reaction conditions. This serves as a control for the methanol-d_4 experiments described in the previous section, ruling out the possibility, albeit remote, that in those

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experiments, deuterium was incorporated into metabolite **3** but subsequently was completely washed out prior to the mass spectrometric analysis.

CONCLUSIONS

The results presented here show that 1,2,4-benzotriazine 1,4-dioxide (**1b**) is able to carry out enzyme-activated, hypoxia-selective DNA strand cleavage analogous to the other benzotriazine di-*N*-oxides that have been characterized.[21,25,43] Two complementary isotopic labeling experiments provided evidence *against* the generation of atom-abstracting drug radicals such as **6** following one-electron reductive activation of 1,2,4-benzotriazine 1,4-dioxide under hypoxic conditions. Accordingly, the data is consistent with a mechanism involving the release of the well-known DNA-damaging agent, the hydroxyl radical from the reductively activated drug intermediate **2b**.

It may be useful to consider how the results described here mesh with the earlier suggestion that spin-trapped adducts of **2** and **6** were detected following microsomal reduction of **1b** in the presence of the nitrone spin-trap PBN (*N*-tert-butylphenyl-α-nitrone).[38] Importantly, recent computational analyses suggested that spin-trapping agents may not serve as "neutral observers" that report on the normal reactions of the bioreductively activated radical intermediate **2**.[28] Rather, spin-trapping agents have the potential to react directly with **2**, in effect, short-circuiting its normal reactivity. Thus, various reactions of spin-trapping agents with **2b**, rather than with **6**, may yield the observed ESR spectra.[28,37,38] It is difficult to rigorously define the structure of a spin-trapped radical using ESR, and further work is required to determine the exact nature of the radicals captured by spin-trapping reagents during the bioreduction of **1b** and other analogues.

Overall, the results are consistent with the hypothesis that bioreductive activation of heterocyclic *N*-oxides under hypoxic conditions leads to the release of the highly cytotoxic reactive species, hydroxyl radical. This molecular mechanism may be relevant to the biological properties of many, structurally varied heterocyclic *N*-oxides under investigation as potential therapeutic agents for the treatment of cancer,[7,61,62] tuberculosis,[63,64] malaria,[65,66] leishmaniasis,[66] Chagas disease,[67] and bacterial infections.[68]

ASSOCIATED CONTENT

Supporting Information

Results of control experiments related to DNA strand cleavage, LC-MS/MS data, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

We thank the National Institutes of Health for partial support of this work (CA 100757).

Notes

The authors declare no competing financial interest.

DEDICATION

This article is dedicated to the memory of Victor Fung, Ph.D., a former Program Officer at NCI and a former Scientific Review Officer of the Cancer Etiology study section of CSR, NIH, for his wisdom, compassion, integrity, his love of sciences and the arts, and, above all, his contributions to the career development of so many investigators during his own distinguished career.

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