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Spin Trapping of Radicals Other Than the •OH Radical upon Reduction of the Anticancer Agent Tirapazamine by Cytochrome P_{450} Reductase

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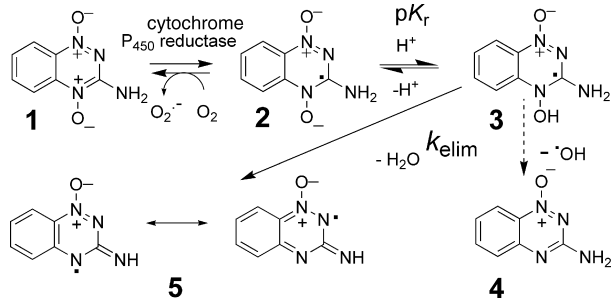
Hypoxia in tumors is a key determinant of resistance to both chemotherapy and radiotherapy treatments and at the same time offers an exploitable target for hypoxia-selective prodrugs (HSPs).[1] The 1,2,4-benzotriazine 1,4-dioxide (BTO) class of HSPs show selectivity for killing hypoxic cells,[2] with tirapazamine (TPZ, **1**) advancing to clinical trials.[3] It is the susceptibility of the radical anion (**2**) and its protonated form (**3**) to back-oxidation by molecular oxygen[4] (Scheme 1) that is pivotal to the hypoxia selectivity of TPZ in killing such cancer cells.

Scheme 1



While there is recognition that the cytotoxin produced upon cellular reduction of the BTO prodrugs is an oxidizing radical,[5] leading to DNA strand breaks and the poisoning of topoisomerase II,[6] the identity of the radical has remained a subject of debate for more than two decades. We have presented spectral and kinetic evidence that **3** undergoes dehydration to the benzotriazinyl radical (BTZ, **5**) (Scheme 1), which is capable of causing oxidative damage to DNA and oxidizing TPZ itself.[7,8] An alternative proposal is that **3** undergoes N−OH bond homolysis to release the •OH radical and the 1-oxide, **4**.[4] The spectrum of products from TPZ-mediated damage to both DNA bases and the deoxyribose sugar has been compared with that arising from the •OH radical.[9] Although the putative TPZ-derived active radical elicits some preference in purine damage over pyrimidine damage, in contrast to the •OH radical, the overall similarity in the range of products has been taken as evidence that the •OH radical is the damaging species. An attempt to identify the radical has been made using the spin trap 5,5′-dimethylpyrroline 1-*N*-oxide (DMPO) combined with electron paramagnetic resonance (EPR) observations.[10] The major signal observed was that of a typical six-line carbon-centered radical with hyperfine coupling constants (HFCs) of $a_N = 15.8$ G and $a_H = 22.3$ G for the DMPO C-centered adduct.[11] This radical may arise from the trapping of **3** or the product formed by reaction of the active radical with materials in the biological matrix. An additional spectrum was also observed in the above study, as well as in an earlier study,[12] with HFCs of $a_N = 15.0$ G and $a_H = 14.9$ G. This spectrum corresponds to the known DMPO−OH radical adduct spectrum,[11] and this finding has been cited as support for the proposal that the •OH radical is produced upon the reduction of TPZ. However, DMPO is known to be subject to one-electron oxidation and to radical addition−elimination reactions, to produce a radical cation that reacts with solvent water to produce the same species as the DMPO−OH radical adduct.[13] In this study, we have employed the nitrone spin-trap 5-diethoxyphosphoryl-5-methyl-pyrroline *N*-oxide (DEPMPO), which is less prone to oxidation than DMPO, as β-phosphorylation raises its oxidation potential relative to that of DMPO.[14]

One-electron reduction of TPZ by cytochrome P_{450} reductase-enriched microsomes[15] was carried out anaerobically at 37 °C within a TE_{011} cavity equipped with a variable-temperature controller (ES-DVT4) on a JEOL JES-FA200 EPR spectrometer[16] operating at 9.1 GHz and 100 kHz field modulation. In agreement with the previous study,[10] using DMPO as the spin trap produced a mixture of two species, (i) a six-line carbon-centered DMPO radical spectrum with $a_N = 16.1$ G, $a_H = 22.5$ G and (ii) a four-line (1:2:2:1) DMPO−OH adduct spectrum with $a_N = 14.8$ G, $a_H = 14.8$ G (Figure 1a).

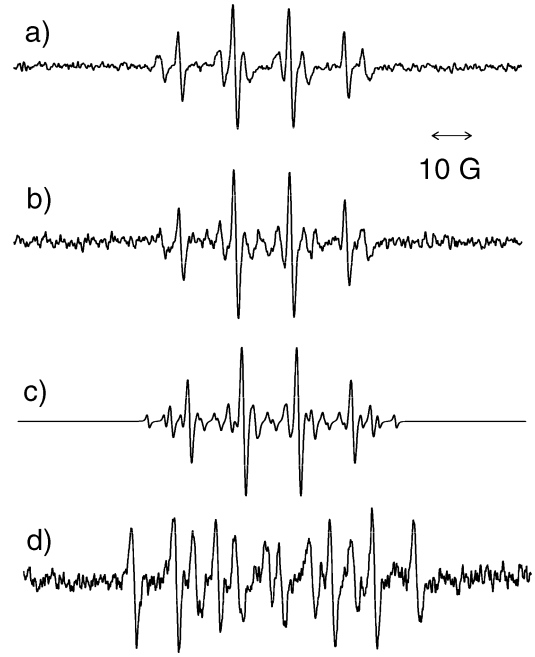


Figure 1. EPR spectra of radicals obtained upon enzymatic reduction of **1** (4 mM) by cytochrome P_{450} reductase-enriched microsomes (2 mg/mL) containing SOD (250 μg/mL), glucose-6-phosphate dehydrogenase (13 units/mL), glucose-6-phosphate (10 mM), and NADPH (1 mM) at pH 7.4 in the presence of (a) DMPO (100 mM) and (b) DMPO in ^{17}O-labeled H_2O (46%) measured at room temperature. (c) Simulation spectrum of DMPO−OH, DMPO−^{17}OH, and DMPO C-centered adducts with a 0.45:0.40:0.15 ratio of radicals ($R = 0.94$). (d) Spectrum in the presence of DEPMPO (25 mM).

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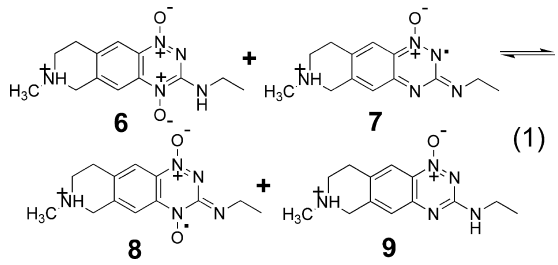
A repeat of the experiment with $H_2{}^{17}O$ (70−75.9 atom % ^{17}O, Isotec) added to give a final isotope content of 46% resulted in an initial spectrum containing a third species (Figure 1b), which was simulated to have HFCs of $a_N = 15.1$ G, $a_H = 14.5$ G, and $a^{17}{}_O = 4.6$ G (Figure 1c). The observed splitting, in addition to those arising from the DMPO−^{16}OH and DMPO C-centered adducts, implies that hydroxylation of the DMPO is sourced from the solvent and not from TPZ. A further experiment was carried out using DEPMPO as the spin trap. The obtained EPR spectrum, with $a_N = 14.7$ G, $a_H = 21.4$ G, $a_P = 47.4$ G, is that for a trapped C-centered radical without any contribution of a DEPMPO−OH adduct species (Figure 1d). The failure in the above experiments to detect radicals other than C-centered radicals may result from factors such as (i) the slow transition from **3** to **5** (k_{elim} of ∼100 s^{-1}),[7] which could favor spin trapping of a C-centered form of **3**; (ii) a slow rate of trapping or short lifetime of a spin-trapped N-centered radical species using DMPO; and (iii) the known reactivity of the BTZ radical **5** in oxidizing the parent benzotriazine 1,4-dioxide **1** to presumably an *N*-oxide radical in a redox equilibrium.[8] In an attempt to overcome these potential drawbacks, an experiment with the highly soluble TPZ analogue *N*-ethyl-7-methyl-6,7,8,9-tetrahydro[1,2,4]triazino[6,5-*g*]isoquinolin-3-amine 1,4-dioxide (**6**) and its 1-oxide derivative, **9**,[17] was carried out using *N*-*tert*-butyl-α-phenylnitrone (PBN) as the spin trap. The high solubility of **9**, in contrast to that of the 1-oxide of TPZ, **4**, can be utilized to maintain a significant concentration of the putative oxidizing radical **7** in equilibrium 1 to aid its spin trapping by PBN. This spin trap has been successfully used in identifying nitrogen-centered radicals, such as in the metabolism of 3-methylindole.[18]



The spectrum observed upon incubation of **6** (640 μM) in the presence of **9** (20 mM) is shown in Figure 2a. It is a composite spectrum simulated (Figure 2b) by spin trapping of a C-centered radical with HFCs of $a_N = 16.00$ G, $a_H = 3.45$ G and a multi-N-centered radical with HFCs of $a_N = 15.70$ G, $a_H = 3.64$ G, $a_N = 2.85$ G, $a_N = 2.14$ G, consistent with the formation of a radical such as a BTZ radical. The involvement of a second N coupling is unusual but most likely arises from the addition of the spin trap to the tautomeric form of the BTZ radical at the N2 position. The N-centered radical decayed faster than the C-centered radical.

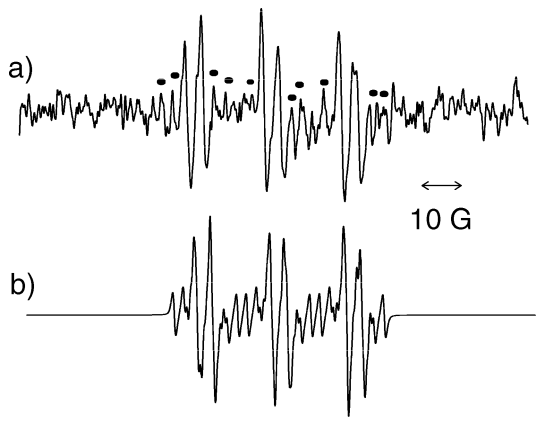


Figure 2. (a) EPR spectra of radicals obtained under the same conditions as in Figure 1 for a 1:30 mixture of compounds **6** and **9** in the presence of the spin trap PBN (50 mM). Dots indicate the multiplicity due to PBN N-centered radical adducts. (b) Simulation spectrum of PBN N- and C-centered radical adducts in the ratio 0.36:0.64 ($R = 0.93$).

In conclusion, we have found evidence that metabolism of the 1,2,4-benzotriazine 1,4-dioxide class of HSPs leads not to the formation of the •OH radical but to both C-centered and N-centered radicals. It is possible that other radical species are produced that are not spin-trapped under the conditions of this study or that the lifetimes of the spin-trapped species are too short for detection by our methodology.

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Supporting Information Available: Methods for preparation of microsomes, solutions for EPR studies, and EPR control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) EPR spectra recorded at a power of 20 mW were averaged (5−10 scans) over a scan range of 200 G with a modulation width of 0.5 G, scan time of 2 min, and time constant of 0.1 ms. Computer simulations of spectra were carried out using the WINSIM EPR program available in the public domain of the NIEHS EPR database. The correlation coefficients, *R*, for all of the spectral simulations were ≥0.93.
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