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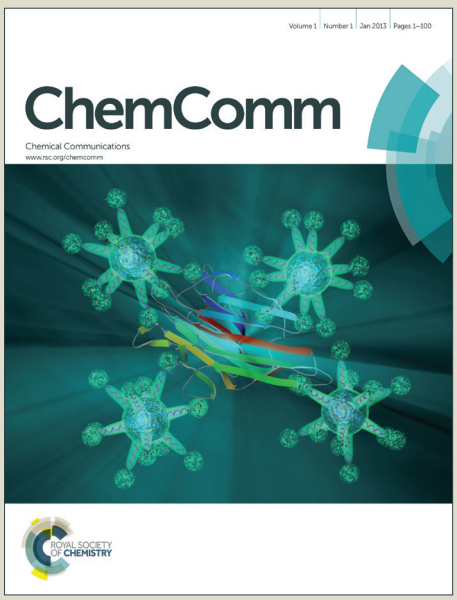

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ARTICLE TYPE

Fragmentation of the quinoxaline *N*-oxide bond to the •OH radical upon one-electron bioreduction

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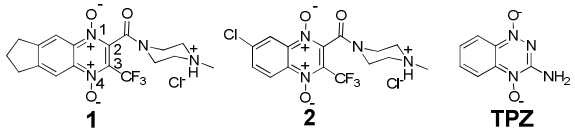
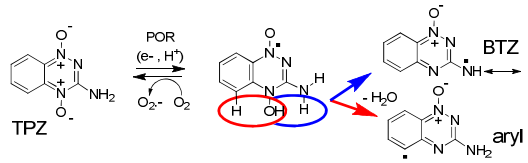
The •OH radical is released from 3-trifluoromethyl-quinoxaline 1,4-dioxides upon one-electron reduction by cytochrome P450 oxidoreductase. This process effectively competes with back oxidation of the intermediate radical anion by oxygen and underlies the increased aerobic cytotoxicity of such compounds compared to that seen for the related clinical bioreductive benzotriazine drug, tirapazamine.



Scheme 1 Structures of quinoxaline compound **1**, **2** and tirapazamine.

Quinoxaline 1,4-dioxide compounds (QDO) are under investigation as bioreductive prodrugs, which upon one-electron reduction, are active against cancer cell lines with the 3-trifluoromethyl derivatives exhibiting considerable cytotoxicity under both oxic and hypoxic conditions. [1] This is in contrast to the related benzotriazine 1,4-dioxides compounds (BTO) which display substantial hypoxia-selectivity in killing treatment-resistant cancer cells while well-oxygenated cells, found in normal tissues, are protected by back oxidation of the initially formed radical anion preventing its breakdown into cytotoxins. [2] For the clinical BTO drug tirapazamine (TPZ, Scheme 1), the cytotoxins include the *N*-centred benzotriazinyl radical (BTZ), which has been identified by EPR, and the C-centred aryl radical, as evidenced by EPR spectra simulations, Scheme 2. [3] Recently,



Scheme 2 Radical pathways upon one-electron reduction of TPZ.

an aryl radical has been spin-trapped upon the one-electron reduction of the 3-alkyl analogue of TPZ, SN30000, using the nitrone spin trap, *N*-*tert*-butyl-α-phenylnitrone (PBN). [3d]

The increased aerobic cytotoxicity of QDO compounds compared to BTO compounds, and their correspondingly low

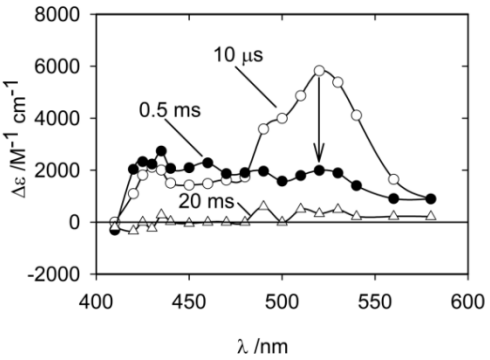


Fig. 1 Time-resolved difference spectra between the radical intermediates and parent compound following one-electron reduction of **1** by the $CO_2^{\bullet-}$ species produced on pulse radiolysis (3 Gy in 200 ns). The solution was saturated with N_2O gas and contained **1** (150 μM), sodium formate (0.1 M) and phosphate buffer (5 mM, pH 7.0). The changes in extinction coefficient were calculated with a radical yield of 0.68 μM Gy^{-1} for $CO_2^{\bullet-}$.

hypoxia-selectivity, is most likely related to the production of different radicals upon one-electron reduction, with the •OH radical being suggested on the basis of DNA strand cleavage and product analysis. [1b] The physical identification of the radical species produced is crucial to the further development of the QDO class as hypoxia-selective cytotoxins. In this study EPR and pulse radiolysis were used to identify and characterise the radicals formed following the one-electron reduction of two 3-trifluoromethyl QDO compounds **1** and **2** (see Supplementary Information for preparation) [1] which bear a methylpiperazine side chain for improved aqueous solubility, Scheme 1.

The compounds were reduced using pulse radiolysis, as previously described for BTO compounds, [3a] yielding radical anions which have an absorption band in the visible range, Fig. 1. These radical anions underwent a kinetic 1st-order transformation to a weakly absorbing transient which decayed on a longer timescale. Fast redox equilibria were established between the one-electron reduced forms of **1** and **2** and the redox indicator benzylviologen, yielding $E(1)$ values of -430 ± 10 mV and -306 ± 10 mV respectively. Due to instability of the radical anions of **1** and **2** (half-life, $t_{1/2}$ *ca.* 100 μs), 0.25 - 0.4 mM concentrations of the compounds and the indicators were used to establish redox equilibria within ca. 5 μs. The $E(1)$ values are higher than that of TPZ (-456 ± 8 mV), [4] which undergoes one-electron reduction by

NADPH-dependent cytochrome P450 oxidoreductase (sPOR). [5] Cloned and highly purified *de novo* human sPOR [3d] was used to reduce **1** and **2** *in situ* in a JEOL JES-FA200 EPR spectrometer equipped with a ES-DVT temperature controller under anaerobic conditions at 310 K in the presence of; the spin trap 5-diethoxyphosphoryl-5-methylpyrroline-1-oxide (DEPMPO), a NADPH regenerating system and both enzymatic and molecular scavengers of possible confounding redox active species.

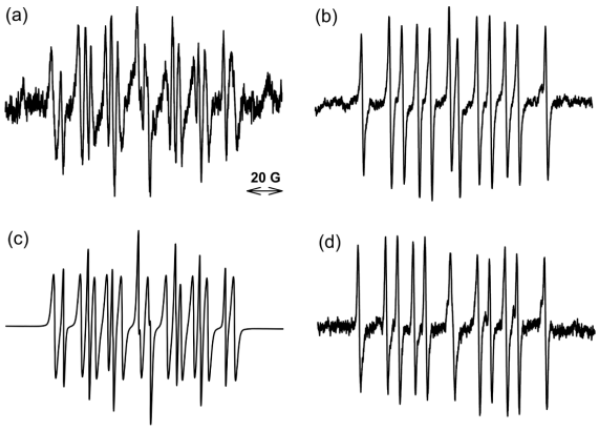
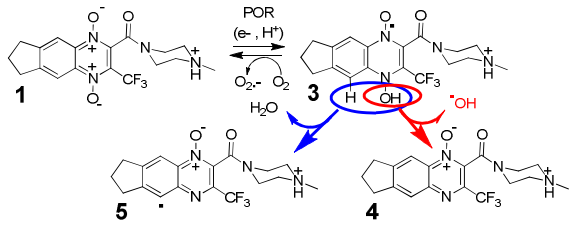


Fig. 2 EPR spectra obtained upon reduction of **1** (5.6 mM (a),(b); 8.1 mM (d)) by sPOR protein (6 ng.ml^{-1}) in solutions at 310 K containing DETAPAC (100 μM), SOD (300 units.ml^{-1}), catalase (1500 units.ml^{-1}), glucose-6-phosphate-dehydrogenase (13 units.ml^{-1}), glucose-6-phosphate (10 mM), and NADPH (1 mM) at pH 7 plus; (a) DEPMPO (25 mM); (b) DEPMPO (25 mM) and DMSO (2 M); (c) simulated spectrum (NIH WinSim software) of DEPMPO-C species (0.84) and DEPMPO-OH (0.16), r = 0.903; (d) DEPMPO (25 mM) and CH_3OH (2 M).

Experiments with **1** gave an initial spectrum with hyperfine coupling constants (HFCs) of aN = 14.0 G, aH =13.2 G, aP = 47.3 G, identifying the DEPMPO-OH species, [6] to which an additional spectrum accumulated over the period of 3 hours with HFCs aN = 14.7 G, aH = 21.3 G, aP = 47.6 G, resulting in a partially overlapping 18-line spectrum, Fig.2(a). The HFCs of the 12-line carbon-centred radical species, DEPMPO-C, do not closely match those reported for a DEPMPO-phenyl species, [7] however HFCs for a trapped aryl radical on a quinoxaline ring have not been reported. Also, these HFCs are the same as for the trapped DEPMPO-C radical formed following the reduction of TPZ. [3b] Simulation of the observed spectrum at 3 hours, Fig. 2(c), indicates that the accumulated spectrum consists of *ca*. 84% DEPMPO-C radical and 16% DEPMPO-OH species. Given the fact that the •OH radical reacts with pyrroline-1-oxide spin traps orders of magnitude faster than with C-centred radicals, [8] this time sequence is expected if both radicals are being formed and trapped. It is possible that the DEPMPO-OH species could arise from the oxidation of DEPMPO by a C-centred radical as it is known that oxidation of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) by aryl radicals, for example, can occur to form DMPO-OH species [9] through inverted spin trapping. [10] To investigate whether the DEPMPO-OH spectrum arises from addition of the •OH radical to DEPMPO or from another pathway, the above experiment was repeated in the presence of dimethylsulphoxide, DMSO (2 M), which reacts rapidly with •OH radicals to release the methyl radical. [11] Both the DEPMPO-OH and DEPMPO-C spectra were replaced by an overall wider 12-line spectrum with HFCs aN = 15.2 G, aH = 21.9 G, aP = 47.7 G, Fig. 2(b), identifying the DEPMPO-CH_3 species. [12] In a further experiment, methanol (2 M) was added in place of DMSO, resulting in a radical with HFCs aN = 15.0 G, aH = 21.4 G. aP = 49.8 G, Figure 2(d), which corresponds to the trapped hydroxymethyl radical, [13] known to arise from H-atom abstraction from methanol by the •OH radical. [14] Analogous EPR spectra were obtained when **2** was used as the substrate, albeit on a shorter timescale due to faster reduction of the more electron-affinic compound by sPOR (see Supplementary Information). Formation of the •OH radical was not detected for QDO analogues containing electron-donating 3-phenyl or 3-methyl substituents (data not shown). The above EPR results confirm that the •OH radical is released upon one-electron reduction of the 3-trifluoromethyl QDO compounds and that the spin-trapped C-centred radicals arise from a subsequent reaction of the •OH radical. Reaction between the •OH radical and the glucose-6-phosphate of the NADPH regenerating system, at a higher concentration of 180 mM, gave rise to a different spectrum, ruling out such a reaction as a source of the observed C-centred radical of the EPR spectrum (see Supplementary Information).



Scheme 3 Formation and breakdown pathways of radical of **1**.

The formation of C-centred radicals on the reduction of **1** and **2** can be thought to arise from the loss of a water molecule from the protonated radical anion to form aryl radicals, as has been proposed for BTO compounds, [3c] Scheme 3. However we have no definitive EPR evidence for aryl radical formation. High concentrations (2 M) of DMSO and methanol must scavenge any •OH radicals formed upon homolytic fragmentation of the N-O bond, preventing radical addition to unsaturated rings as another possible source of C-centred radicals. DFT calculations were undertaken to investigate the feasibility of different breakdown pathways following protonation of the radical anion on N4-O, e.g. **3** in Scheme 3, by calculating the differences in overall energy for (i) their homolytic fragmentation to form their 1-oxides, e.g. **4**, with release of the •OH radical, and (ii) formation the aryl radicals centred at C5, radical **5**, through water elimination, Scheme 3. The calculations using Gaussian 09 software [15] with water simulation, based on the polarized continuum model (IEFPCM), [16] showed both pathways (i) and (ii) to be more exothermic for **3** compared to the radical anion of TPZ, with Δ*G* values (i) -2.6 kcal mol^{-1}, (c.f. +2.2 kcal mol^{-1}) and (ii) -6.5 kcal mol^{-1}, (c.f. +0.5 kcal mol^{-1}). [3c] Protonation of the radical anion on N1-O, followed by similar breakdown pathways, proved to be much less exothermic (see Supplementary Information). BTO compounds with strong electron withdrawing groups, such as the trifluoromethyl moiety in the 3-position, have

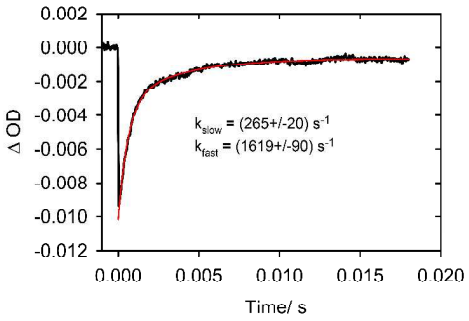


Fig. 3 Example change in transmittance at 520 nm following pulse radiolysis of the solution in Fig.1. The data is fitted to the expression for two consecutive 1st-order reactions (red line).

not been synthesised, so a direct comparison of such a BTO radical anion with **3** can not be made. However, the radical anion of TPZ, which possess an alpha-H on the 3-NH_2 substituent, predominately undergoes a water elimination to form a BTZ radical, [3a] with a calculated ΔG values of -10.9 kcal mol^{-1}, and based on spectra simulation, a minor amount of an aryl radical. [3c]

The demonstrated release of the •OH radical from the radical anion of **1** and **2** in this study is a mechanistic lead in the search for potent bioreductive anticancer prodrugs, but its release can be associated with loss of hypoxia-selectivity. [1] Release of the •OH radical is unlikely to be only factor determining the poor hypoxia-selective cytotoxicity of the 3-trifluoromethyl QDO compounds compared to BTO compounds of similar $E(1)$. [1, 4b] The radical anions of nitroaromatic compounds in general undergo a back oxidation reaction with molecular oxygen with a 2nd-order rate constant, kO_2, according to the relationship; $\log kO_2$ /M^{-1} s^{-1} = 4.6 – 5 × $E(1)$ /V. [17] Thus for the radical anions of TPZ and **1** under aerobic conditions (0.26 mM [O_2]), the calculated pseudo 1st-order rate constants are 1970 s^{-1} and 1460 s^{-1} respectively. The 1st-order rate constant for the back oxidation of the radical anion of TPZ is sufficiently greater than the rate constant for the formation of the oxidising BTZ radical on the breakdown of the radical anion of TPZ of 83 s^{-1} at pH 7, [17b] thus ensuring good hypoxia-selectivity. The radical anion of **1** undergoes a biphasic transformation, Fig. 3, at 1900 ± 200 s^{-1} and 330 ± 20 s^{-1} (average of 10 determinations). The initial phase is not associated with •OH radical release as an absorbing transient is formed, whereas the second phase may well be. The combination of a significant amount of released •OH radical in the presence of oxygen most likely accounts for the observed aerobic cytotoxicity.

In conclusion, the •OH radical is released from 3-trifluoroquinoxaline 1,4-dioxides upon one-electron reduction. This process is suggested to underlie the observed enhanced aerobic cytotoxicity of such compounds compared to other bioreductive drugs such as the BTO drug, tirapazamine. Thus this study underlines the need to consider both kinetic and redox factors in the development of hypoxia-selective bioreductive drugs which act by a radical mechanism.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis of quinoxaline 1,4-dioxide compounds **1** and **2**; Pulse radiolysis methods; EPR, spectra of compound **2** and control experiments; DFT information, including full reference 15 in the text. See DOI: 10.1039/b000000x/

1. (a) M. M. F. Ismail, K. M. Amin, E. Noaman, D. H. Soliman and Y. A. Ammar, *Eur. J. Med. Chem.*, 2010, **45**, 2733-2738; (b) V. Junnotula, A. Rajapakse, L. Arbillaga, A. López de Cerain, B. Solano, R. Villar, A. Monge and K. S. Gates, *Bioorg. Med. Chem.*, 2010, **18**, 3125-3132; (c) B. Solano, V. Junnotula, A. Marin, R. Villar, A. Burguete, E. Vicente, S. Perez-Silanes, I. Aldana, A. Monge, S. Dutta, U. Sarkar and K. S. Gates, *J. Med. Chem.*, 2007, **50**, 5485-5492.
2. (a) K. Laderoute, P. Wardman and A. M. Rauth, *Biochem. Pharmacol.*, 1988, **37**, 1487-1495; (b) M. A. Baker, E. M. Zeman, V. K. Hirst and J. M. Brown, *Cancer Res.*, 1988, **48**, 5947-5952; (c) K. A. Biedermann, J. Wang, R. P. Graham and J. M. Brown, *Br. J. Cancer*, 1991, **63**, 358-362; (d) J. M. Brown and L.-H. Wang, *Anti-Cancer Drug Des.*, 1998, **13**, 529-539.
3. (a) R. F. Anderson, S. S. Shinde, M. P. Hay, S. A. Gamage and W. A. Denny, *J. Am. Chem. Soc.*, 2003, **125**, 748-756; (b) S. S. Shinde, M. P. Hay, A. V. Patterson, W. A. Denny and R. F. Anderson, *J. Am. Chem. Soc.*, 2009, **131**, 14220-14221; (c) S. S. Shinde, A. Maroz, M. P. Hay, A. V. Patterson, W. A. Denny and R. F. Anderson, *J. Am. Chem. Soc.*, 2010, **132**, 2591-2599; (d) R. F. Anderson, P. Yadav, D. Patel, J. Reynisson, S. R. Tipparaju, C. P. Guise, A. V. Patterson, W. A. Denny, A. Maroz, S. S. Shinde and M. P. Hay, *Org. Biomol. Chem.*, 2014, **12**, 3386-3392.
4. (a) K. I. Priyadarsini, M. Tracy and P. Wardman, *Free. Rad. Res.*, 1996, **25**, 393-400; (b) M. P. Hay, S. A. Gamage, M. Kovacs, F. B. Pruijn, R. F. Anderson, A. V. Patterson, W. R. Wilson, J. M. Brown and W. A. Denny, *J. Med. Chem.*, 2003, **46**, 169-182.
5. A. V. Patterson, M. P. Saunders, E. C. Chinje, L. H. Patterson and I. J. Stratford, *Anti-Cancer Drug Design*, 1998, **13**, 541-573.
6. R. J. Singh, H. Karoui, M. R. Gunther, J. S. Beckman, R. P. Mason and B. Kalyanaraman, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 6675-6680.
7. B. C. Gilbert, P. Ionita, J. R. L. Smith, J. Oakes and N. Ouwerkerk, *ARKIVOC*, 2006, 127-147.
8. (a) E. Finkelstein, G. M. Rosen and E. J. Rauckman, *J. Am. Chem. Soc.*, 1980, **102**, 4994-4999; (b) H. Taniguchi and K. P. Madden, *J. Am. Chem. Soc.*, 1999, **121**, 11875-11879.
9. K. J. Reszka and C. F. Chignell, *Chem.-Biol. Interact.*, 1995, **96**, 223-234.
10. K. Ranguelova and R. P. Mason, *Magn. Reson. Chem.*, 2011, **49**, 152-158.
11. W. T. Dixon, R. O. C. Norman and A. L. Buley, *J. Chem. Soc.*, 1964, 3625-3634.
12. H. Karoui, F. Chalier, J.-P. Finet and P. Tordo, *Org. Biomol. Chem.*, 2011, **9**, 2473-2480.
13. C. Frejaville, H. Karoui, B. Tuccio, F. Le Moigne, M. Culcasi, S. Pietri, R. Lauricella and P. Tordo, *J. Med. Chem.*, 1995, **38**, 258-265.
14. K.-D. Asmus, H. Mockel and A. Henglein, *J. Phys. Chem.*, 1973, **77**, 1218-1221.
15. Gaussian 09, *revision A.02* ed. *Frisch, M. J. et al., Gaussian Inc., Wallingford CT, 2009.*
16. J. Tomasi, B. Mennucci and R. Cammi, *Chem. Rev.*, 2005, **105**, 2999-3093.
17. (a) P. Wardman, M. F. Dennis, S. A. Everett, K. B. Patel, M. R. L. Stratford and M. Tracy, *Biochem. Soc. Symp.*, 1995, **61**, 171-194. (b) R. F. Anderson, S. S. Shinde, M. P. Hay, S. A. Gamage and W. A. Denny, *Org. Biomol. Chem.*, 2005, **3**, 2167-2174.