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The One-Electron Reduction Potential of 3-Amino-1,2,4-benzotriazine 1,4-dioxide (Tirapazamine): A Hypoxia-Selective Bioreductive Drug

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The one-electron reduction potential of 3-amino-1,2,4-benzotriazine 1,4-dioxide, tirapazamine (SR 4233) in aqueous solution has been determined by pulse radiolysis. Reversible electron transfer was achieved between radiolytically-generated one-electron reduced radicals of tirapazamine (T), and quinones or benzyl viologen as redox standards. The reduction potential $E_{m7}(T/T^{\bullet-})$ was -0.45 ± 0.01 V vs. NHE at pH 7. From the pH dependence of the reduction potential, $pK_a = 5.6 \pm 0.2$ was estimated for the tirapazamine radical, a value similar to the pK_a determined by other methods.

Keywords: Tirapazamine, reduction potential, pK_a, pulse radiolysis

INTRODUCTION

Tirapazamine, 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233) is an anti-tumour agent with selective toxicity towards hypoxic cells, and is in Phase I clinical trial.[1–5] As the stable products of reduction are not biologically active it is believed that the one-electron reduced intermediate is involved in inducing DNA strand breaks.[2] The selective hypoxic toxicity involves metabolic reduction of the drug, with electron transfer to oxygen in normal (well oxygenated) cells, when the drug radical reverts back to the parent. Previous studies have focused on the reduction mechanism of tirapazamine using pulse radiolytic, electrochemical and enzymatic reductions.[2,6–8] Some key chemical properties of its free radicals in water have been measured,[2,7] but the one-electron reduction potential of the compound has not been reported. Tocher *et al.* used electrochemical methods to determine the reduction potential in aprotic solvents.[6–8] Redox properties are influenced by the dielectric constant of the medium and hence these measure-

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ments cannot be confidently extrapolated to aqueous solution.

Pulse radiolysis is a reliable method to measure reduction potentials involving unstable free radicals by quantifying the positions of electron-transfer equilibria.[11–13] In this study, we present measurements on the one-electron reduction potential of tirapazamine in water using known redox indicators, and of the effect of pH on this potential. The latter provides an important independent evaluation of radical pK_a, which was needed to resolve a discrepancy in our mechanistic studies. Thus two types of pulse radiolysis data had yielded $pK_a = 6.0$ for the tirapazamine radical,[2] whereas the effects of pH on the radiolytic chain reduction with H-donors had shown an apparent 'pK_a' 1–2 pH units higher.[9,10]

MATERIALS AND METHODS

Materials

Tirapazamine was synthesized at the Bio-Organic Chemistry Laboratory, SRI International. Benzyl viologen and duroquinone were from Sigma. Anthraquinone sulphonic acid, sodium salt (BDH) was recrystallized from water. All solutions were prepared in water from a 'Milli-Q' system (Millipore). Phosphate salts (5 mmol dm^{-3}) were used to adjust the pH with NaOH and $HClO_4$ (Merck) when required. Gases (N_2O, O_2 and N_2O/O_2 mixtures) were from British Oxygen Co. All experiments were carried out at room temperature.

Methods

For the pulse radiolysis experiments, electron pulses (30 ns, 3.5 MeV) from a van de Graaff accelerator, with typical absorbed doses of 2–3 Gy, were used for most of the studies. The absorbed dose was determined using N_2O-saturated 10 mmol dm^{-3} thiocyanate, monitoring $(SCN)_2^{\bullet-}$ at 472 nm assuming the product of yield and extinction coefficient was 5.06×10^{-4} m^2 mol^{-1}. The radicals were detected spectrophotometrically using 2 cm pathlength. Pulse radiolysis methodology at the Gray Laboratory has been previously described.[14] 2-Propanol radicals were used as a reductant to generate the one-electron reduced radicals of tirapazamine (T), using N_2O-saturated 0.5 mol dm^{-3} 2-propanol and reaction with radiolytically-produced primary radicals, $H^{\bullet}$ and $^{\bullet}OH$:[15]

$$H^{\bullet}/{}^{\bullet}OH + (CH_3)_2CHOH \rightarrow H_2/H_2O + (CH_3)_2C^{\bullet}OH \quad (1)$$

$$T + (CH_3)_2C^{\bullet}OH \rightarrow T^{\bullet-} + CH_3COCH_3 + H^+ \quad (2)$$

The ionic strength of the solutions was kept low (~0.01 mol dm^{-3}) to minimise the effect of ionic strength on the measurements. Data analysis used Origin software (Microcal).

RESULTS

Redox Equilibria and Reduction Potentials

In a solution containing tirapazamine and a redox indicator (R: either duroquinone (DQ), anthraquinone sulphonate (AQS^-) or benzyl viologen (BV^{2+})), the initial radical distribution depends on kinetic competition, i.e. essentially by the ratio of the concentration of the two solutes as the rate constants of these compounds with 2-propanol radicals are nearly equal. Radical generation is followed by rapid thermodynamic equilibrium between the two partners in a few microseconds:

$$T^{\bullet-} + R \rightleftharpoons T + R^{\bullet-} \quad (3)$$

The equilibrium constant K_3 was calculated from the absorbance changes (A) at convenient wavelengths as:

$$A_{eq} = [A_T + K_3 A_R([R]/[T])]/[1 + K_3([R]/[T])] \quad (4)$$

where A_T and A_R are the absorbances of the individual radicals T and R respectively, and A_{eq} is

that with the mixture of T and R at equilibrium.[11–13,16,17] The equilibrium constant K_3 was estimated by fitting the data (A_{eq} vs. [R]/[T]) by non-linear least-squares to equation (4), and is related to the reduction potentials by:[13]

$$\Delta E = E(\mathrm{R/R^{\bullet -}}) - E(\mathrm{T/T^{\bullet -}}) = (RT/F)\ln K_3 \quad (5)$$

Quinones (Q) and viologens (V^{2+}) are convenient redox indicators (R), as their reduction potentials are known and the radicals exhibit strong absorption, with their absorption maxima at 440 nm for radicals from duroquinone ($DQ^{\bullet -}$), 490–500 nm for 9,10-anthraquinone-2-sulphonate ($AQS^{\bullet 2-}$), and 600 nm for benzyl viologen ($BV^{\bullet +}$).[16,17]

The reversibility of equilibrium was confirmed by analysing the rate of decay of the tirapazamine radical and formation of the quinone/viologen radicals. Assuming no significant net radical loss during the establishment of equilibrium (valid with the radical concentrations used), the rate of approach is characterized by a first-order rate constant (k_{obs}):[13]

$$k_{obs} = k_f[R] + k_r[T] \text{ or } k_{obs}/[R] = k_f + k_r([T]/[R]) \quad (6)$$

where k_f and k_r are the rate constants for the forward and reverse reactions in equilibrium (3). The intercept and slope of the linear plot of k_{obs}/[R] vs. [T]/[R] correspond to k_f and k_r respectively and the equilibrium constant $K_3 = k_f/k_r$.

Redox Equilibria with Tirapazamine and Anthraquinone Sulphonate

Redox equilibrium was achieved between tirapazamine and AQS^-, 30–50 μs after pulse radiolysis of solutions containing 2-propanol/N_2O, tirapazamine (25–400 μmol dm^{-3}) and AQS^- (15–60 μmol dm^{-3}). In the absence of AQS^-, tirapazamine radicals decay by second-order kinetics over a few milliseconds: the first half-life of the radicals at initial concentrations of ~4 μmol dm^{-3} is ~8 ms at pH 7.4. In the presence of AQS^-, their decay was much faster (a few microseconds) and changed to first-order kinetics with simultaneous formation of $AQS^{\bullet 2-}$ at 500 nm. The transient decay at 540–560 nm and the formation at 500 nm were not only dependent on the concentration of tirapazamine, but also on AQS^-, confirming the reversibility of the reaction. Absorbances (A_{eq}) at 560 nm and 500 nm were measured after 40–50 μs. The absorbance changes A_{eq} at 500 nm and 560 nm varied with [AQS^-]/[T] and were fitted to equation (4), yielding K_3 as 10.9 ± 1.3 and 11.5 ± 1.7 respectively. An example of the data is given in Figure 1. Analysis of the rates of decay (k_{obs} at 540 nm) in the presence of variable concentrations of AQS^- and tirapazamine according to equation (6) gave K_3 = 10.5 ± 1.7 (Figure 2). The rate constants for the forward and backward reactions estimated from Figure 2 are listed in Table 1. An average value of K_3 = 11.0 ± 0.9, from the above three independently-determined values, was used to calculate ΔE = 62 ± 3 mV from equation (5). Using $E(AQS^-/AQS^{\bullet 2-})$ = −0.396 V at pH 7,[17] the reduction potential of tirapazamine at pH 7 was estimated as E_{m7} ($T/T^{\bullet -}$) = −0.46 ± 0.01 V vs. NHE.

Redox Equilibria with Tirapazamine and Duroquinone

In a solution containing 2-propanol/N_2O at pH 7, tirapazamine (2 mmol dm^{-3}) and duroquinone (10–50 μmol dm^{-3}), the radicals produced initially were mainly from tirapazamine, but an equilibrium was established in a few microseconds. Observation of the absorption by $DQ^{\bullet -}$ at 440nm was made difficult by the strong absorption by the ground state of tirapazamine, and measurements were therefore restricted to 560 nm where tirapazamine radicals absorb. Absorbance changes at 560 nm after 40–50 μs analysed according to equation (4) gave K_3 as 1200 ± 200. The observed first-order rate constants at 560 nm fitted to equation (6) yielded k_f and k_r (Table 2) and the equilibrium constant K_3 = 1050 ± 500. An average value of 1125 ± 270 was used to determine

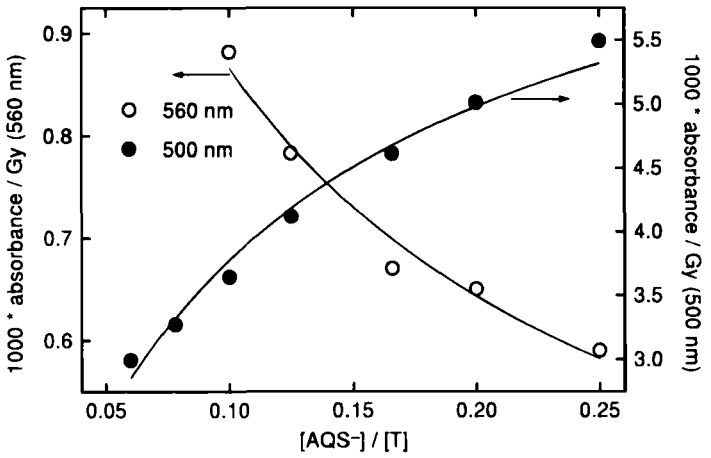

FIGURE 1 Electron-transfer equilibrium between anthraquinone sulphonate (AQS^-) and tirapazamine (T) at pH 7. Change in absorbance (after 40 μs) at 560nm (○) and 500 nm (●) with the ratio of $[AQS^-]/[T]$ were fitted to equation (4) to obtain the equilibrium constant K_3.

$\Delta E = 180 \pm 7$ mV. Using $E(DQ/DQ^{\bullet-}) = -0.264$ V at pH 7,[17] the reduction potential for tirapazamine $= -0.44 \pm 0.01$ V vs. NHE.

Redox Equilibria with Tirapazamine and Benzyl Viologen

Redox equilibrium between tirapazamine (0.4–0.8 mmol dm^{-3}) and BV^{2+} (0.04–0.1 mmol dm^{-3}) yielded estimates of K_3 using the absorption and the kinetic methods as 22 ± 1 and 26 ± 6 mV respectively. An average value of 24 ± 3 mV gives ΔE as 82 ± 6 mV; using $E(BV^{2+}/BV^{\bullet+}) = -0.374$ V,[17] $E(T/T^{\bullet-})$ was estimated to be -0.46 ± 0.01 V.

The reduction potential of tirapazamine determined using the three indicators was similar within experimental uncertainties; an average value of –0.45 ± 0.01 V vs. NHE is recommended

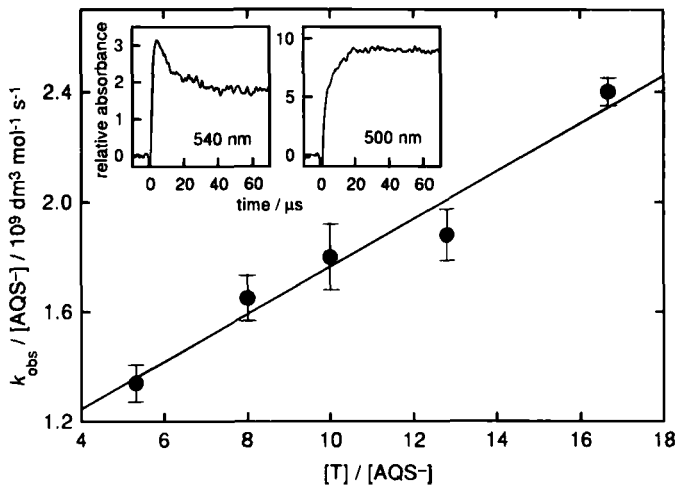

FIGURE 2 Dependence of the observed first-order decay rate constant measured at 540nm (k_{obs}) on the concentrations of anthraquinone sulphonate (AQS^-) and tirapazamine (T). Inset: transient absorptions at 540 and 500nm in a solution containing 60 μmol dm^{-3} AQS^- and 320 μmol dm^{-3} tirapazamine, 0.5 mol dm^{-3} 2-propanol/N_2O at pH 7.

TABLE I Electron Transfer Equilibrium with Tirapazamine at pH 7 ($T^{\bullet-} + R \rightleftharpoons T + R^{\bullet-}$)

R	$E(R/R^{\bullet-})/V$[a]	$10^{-9}\,k_f$[b]	$10^{-8}\,k_r$[b]	K_3 (absorbance)[c]	K_3 (kinetics)[d]	ΔE/mV
AQS^-	−0.396	0.90 ± 0.14	0.86 ± 0.02	11.2 ± 1.1	10.5 ± 1.7	62 ± 3
DQ	−0.260	2.1 ± 0.1	0.02 ± 0.01	1200 ± 200	1050 ± 500	180 ± 7
BV^{2+}	−0.374	4.1 ± 0.3	1.6 ± 0.3	22 ± 1	26 ± 5	82 ± 6

[a]Reference 17. [b]$dm^3\ mol^{-1}\ s^{-1}$. [c]Equation (4). [d]Equation (6).

as the one-electron reduction potential of tirapazamine at pH 7, $E_{m7}(T/T^{\bullet-})$. Ionic strength effects on the equilibria were ignored as they would be of the same order as the uncertainties of the measurements.

Effect of pH on Reduction Potential

The reduction potential of the tirapazamine/radical couple varies with pH, reflecting two half-cells:

$$T + e^- = T^{\bullet-} \quad (7)$$

$$T + H^+ + e^- = TH^{\bullet} \quad (8)$$

linked by the prototropic equilibrium:

$$TH^{\bullet} \rightleftharpoons T^{\bullet-} + H^+ \quad (9)$$

If $K_9 << 1$, the mid-point potential of the half-cell (E_{mi}) at pH_i varies with pK_9 as:[13]

$$E_{mi} \approx E_o + 0.059 \log(10^{-pK9} + 10^{-pHi}) \quad (10)$$

if potentials are in volts, where E_o is the potential of the couple $E(T,H^+/TH^{\bullet})$ at the standard state of unit activities (pH 0). To study the pH effect on the reduction potential, BV^{2+} was used as the redox standard as its potential is independent of pH over the range of interest. The redox equilibrium was established between the radicals of BV^{2+} and T in 20–40 μs and the absorbance changes were monitored at 600 nm. The equilibrium constants were determined at different pH values, adjusting the concentration differentials appropriately (Table 2). The estimated reduction potentials (E_{mi}) were fitted to equation (10), yielding $pK_9 = 5.6 \pm 0.2$ and $E_o = -0.12 \pm 0.01$ V vs. NHE, as shown in Figure 3. Effects of ionization of tirapazamine ground state ($pK_a \sim 12.5$)[2] on the reduction potential at pH < 10 are not significant.

DISCUSSION

The bioreductive activation of the drug is characterised by the thermodynamic parameter, reduction potential, which controls the relative ease of reduction and also other reactions with one-electron acceptors like oxygen. By measuring the equilibrium constants of electron-transfer equilibria before unwanted radical/radical reactions occur, we have estimated the one-electron reduction potential of tirapazamine at pH 7 in aqueous solution as −0.45 V vs. NHE. (This value explains

TABLE II Effects of pH on the Electron-Transfer Equilibrium Between Tirapazamine and BV^{2+} ($T^{\bullet-} + BV^{2+} \rightleftharpoons T + BV^{\bullet+}$)

pH	$[BV^{2+}]$/μmol dm^{-3}	[T]/μmol dm^{-3}	K_3	ΔE/mV
3.1	400–800	40–100	0.09 ± 0.01	−62 ± 3
4.0	50–300	100–200	1.0 ± 0.1	0 ± 3
4.9	40–100	100–400	1.9 ± 0.2	16 ± 3
6.0	50–200	600	9.9 ± 1.6	59 ± 4
7.0	40–100	400–800	22 ± 1	79 ± 1
9.0	40–80	600–800	18 ± 2	74 ± 3

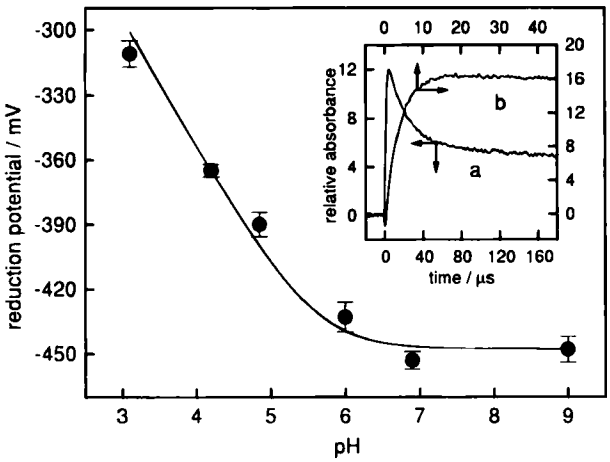

FIGURE 3 Effect of pH on the one-electron reduction potential of tirapazamine calculated from the redox equilibrium between tirapazamine and benzyl viologen. The line shows the fit to equation (10). The error bars reflect four measurements fitted to equation (4). Inset: transient absorption signals at 600 nm in solutions containing (a) 100 μmol dm^{-3} BV^{2+} and 200 μmol dm^{-3} tirapazamine at pH 4 (left/bottom scales), and (b) 40 μmol dm^{-3} BV^{2+} and 800 μmol dm^{-3} tirapazamine at pH 7 (right/top scales).

our earlier failure to observe easily electron transfer between tirapazamine and methyl viologen, since the two potentials are essentially identical.) Thus the one-electron reduction potential of tirapazamine in water is bracketed by those of the nitroimidazole radiosensitizers misonidazole (–0.40 V) and metronidazole (–0.50 V).

Since the tirapazamine radical is unstable in aqueous solution, conventional polarographic methods cannot be easily used to estimate the one-electron reduction potential, and electrochemical potentials in aprotic solvents (where the radical is more stable) do not equate to those in water, primarily because of solvation effects. Even mixed aqueous solvents introduce significant and variable changes: the reduction potential of an uncharged 2-nitromidazole (misonidazole) *decreases* from –0.40 V in water to –0.45 V in ethanol:water (1:1 v/v), whereas that of methyl viologen *increases* from –0.45 V to –0.36 V in ethanol:water (1:1 v/v).[18,19] Voltammetric measurements in dimethyl formamide and acetonitrile yielded estimated half-wave potentials of ~–1.03 V vs. the Ag/AgCl electrode for tirapazamine.[8] On the hydrogen scale (vs. NHE) this value is ~–0.80 V neglecting any effects of liquid junction potentials. The reduction potentials of simple quinone/semiquinone couples in water vs. NHE are ~0.47 V more positive than half-wave potentials in dimethyl formamide vs. SCE electrode,[20] or around 0.23 V more positive after allowing for the difference in the reference electrodes. Change from an aprotic solvent to water therefore shows marked differences in the reduction potentials.

The measured reduction potential varies with pH below pH ~6, because of the involvement of a prototropic equilibrium involving the radical. The potential increases by ~0.06 V for each unit of pH below the radical pK_a. From the pH dependence, $pK_a = 5.6 \pm 0.2$ was estimated. Laderoute *et al.* estimated the radical pK_a = 6.0 at ionic strength 0.1–0.2 from two independent measurements of the pH-dependent radical absorption or decay kinetics.[2] The estimate of radical pK_a obtained in the present study using a third method is in reasonable agreement. The chain reaction (higher than expected reduction efficiency) observed on one-electron reduction of tirapazamine in the presence of hydrogen donors such as formate, 2-propanol or deoxyribose is pH-dependent.[2,9,10] The chain length is higher at

lower pH,[2] with a point of inflection (apparent 'pK_a') around 7.3–7.8.[9,10] While this suggests that the protonated radical is more reactive in chain propagation (presumably hydrogen abstraction) than the radical-anion, the pK_a for dissociation for the protonated tirapazamine radical has been confirmed by the present measurements to be close to 6.0 rather than in the range 7.3–7.8. Thus the chain reduction process cannot be interpreted simply in terms of more efficient hydrogen abstraction by the protonated radical: other pH-sensitive processes must be involved.

Acknowledgments

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