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Chemical properties which control selectivity and efficacy of aromatic *N*-oxide bioreductive drugs

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Summary Pulse radiolysis was used to generate radicals from one electron reduction of 1,2,4-benzotriazine-1,4-dioxides (derivatives of tirapazamine), and of imidazo[1,2-*a*]quinoxaline-4-oxides (analogues of RB90740), which have selective toxicity towards hypoxic cells. Radicals from the mono *N*-oxides (from the latter compounds) react with oxygen ~10-40 times faster than does the tirapazamine radical. Radicals from the tirapazamine analogues studied react with oxygen up to ~10 times slower than tirapazamine radicals. The quinoxaline *N*-oxide radicals are involved in prototropic equilibria with pK_a values (5.5 to 7.4) spanning that reported for tirapazamine (6.0). Generation of radicals radiolytically in the presence of H donors (formate, 2-propanol, deoxyribose) indicate a chain reaction ascribed to H abstraction by the drug radical. The protonated drug radical is much more reactive than the radical anion (H abstraction rate constant $\approx 10^2-10^3$ dm^3 mol^{-1} s^{-1}). Chain termination is ascribed to drug radical-radical reactions, i.e. radical stability in anoxia, with rate constants $2k \approx 1\times10^7$ to 2×10^8 dm^3 mol^{-1} s^{-1} at pH ~7.4. Estimates of the reduction potentials of the drug-radical couples in water at pH 7 for two of the mono-*N*-oxides were in the range -0.7 to 0.8 V *vs* NHE at pH 7.

Keywords: *N*-oxides; tirapazamine; RB90740; radicals; rate constants; hydrogen abstraction

Tirapazamine (SR4233, Figure 1) is a benzo-1,2,4-triazine-1,4-di-*N*-oxide showing selective toxicity towards hypoxic cells (Zeman *et al.*, 1986). The partially reduced product, the 1-oxide (SR4317) shows little toxicity. In contrast, some mono-*N*-oxides based on the imidazo[1,2-α]quinoxaline moiety such as RB91724 (Figure 1) show broadly similar selective toxicity towards hypoxic cells to tirapazamine (Naylor *et al.*, 1993). The electron-adduct (radical anion) of tirapazamine was shown to react rapidly with oxygen (Laderoute *et al.*, 1988), and thus the basis for the selective toxicity (i.e. protection by oxygen) could be reasonably ascribed to the activation of the drug by reductive metabolism to the radical anion, followed by fast restoration of the drug by oxygen:

drug + reducing agent + enzyme → drug radical (1)

drug radical + oxygen → drug + superoxide radical (2)

Because of the low toxicity of further reduction products (Zeman *et al.*, 1986) and evidence for DNA damage induced in model systems where the radical was produced (Laderoute *et al.*, 1988) [*cf* (Naylor *et al.*, 1994)], it was hypothesised that the tirapazamine radical was the active species (Laderoute *et al.*, 1988). However, no quantitative data for radical reactivity, other than the rate constants for reaction (2) and the disproportionation reaction (3) (varying with pH), together with the equilibrium constant pK_4, were reported.

2 drug radicals → reduction product(s) (3)

protonated drug radical ⇌ drug radical + H^+ (4)

We summarise here experiments which facilitate comparison between the properties of the free radicals obtained on reduction of tirapazamine, some analogues substituted in the 3-position (Figure 1) and three representative imidazoquinoxaline mono-*N*-oxides (Figure 1). The chemical properties are compared with the reported cytotoxicity data (Zeman *et al.*, 1986, 1989; Minchinton *et al.*, 1992; Naylor *et al.*, 1993, 1994).

Materials and methods

Tirapazamine and its analogues were synthesised by the Bio-Organic Chemistry Laboratory, SRI International; RB compounds were synthesised at the MRC Radiobiology Unit. Other chemicals were the highest available purity from BDH/Merck Ltd or Sigma. Gases were from British Oxygen Co. The pulse radiolysis apparatus has been described (Candeias *et al.*, 1993). HPLC analysis of reduction products used a base-

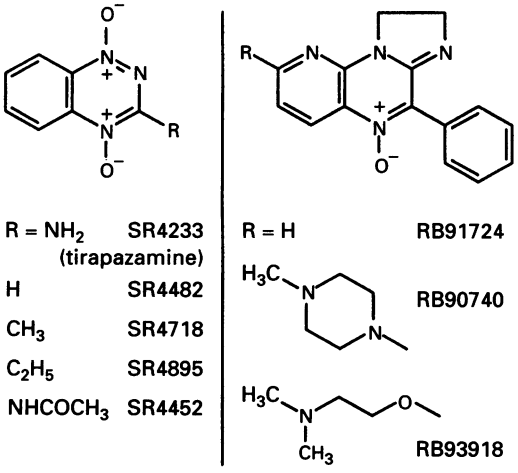

Figure 1 Structures of the compounds studied.

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deactivated reversed-phase column (Hichrom RPB, 100 × 4.6 mm) and linear gradients of phosphate buffers and acetonitrile. Detection was by absorbance using Waters 486 or 996 detectors. Heptane sulphonic acid was included as ion pairing agent in some experiments to improve resolution. Some compounds showed absorbance changes with pH, assigned to prototropic equilibria:

$$\text{protonated drug} \rightleftharpoons \text{drug} + H^+ \quad (5)$$

which were measured using a Hewlett-Packard 8452A spectrophotometer. Values of pK_4 and pK_5 were derived by non-linear least-squares fit of the appropriate function using Origin software on a PC (Microcal).

Results

Generation of drug radicals by pulse radiolysis and their prototropic properties

Pulse radiolysis of solutions of drug (typically ~50 μmol dm^{-3}) in aqueous solutions of sodium formate (0.1 mol dm^{-3}) saturated with nitrous oxide yielded drug radicals in a few microseconds (Wardman, 1992). The reducing agent is $CO_2^{\bullet-}$, reacting to produce the radical at rates near the diffusion-controlled limit:

$$CO_2^{\bullet-} + \text{drug} \rightarrow \text{drug radical} \quad (6)$$

Thus the three RB compounds showed $k_6 \sim 1-4 \times 10^9$ dm^3 mol^{-1} s^{-1} [*cf* tirapazamine, $k_6 = 2.8 \times 10^9$ dm^3 mol^{-1} s^{-1} (Laderoute *et al.*, 1988)]. The absorption spectra of the radicals overlapped those of the parent drug so that at some wavelengths radiolysis induced a negative absorption (bleaching), where the extinction coefficient of the radical was lower than that of the drug. Thus spectral changes broadly similar to those reported for tirapazamine reduction to the radical were obtained. Most measurements were made in the 450–550 nm region where there was little interference from the drugs' ground state absorptions.

Absorption spectra of the radicals of the RB compounds were measured at pH 4, 7 and 11 to search for prototropic equilibria such as (4), for which pK_4 = 6.0 in the case of tirapazamine. Spectral changes were observed broadly similar to those reported for tirapazamine and consistent with equilibrium (4), and values of pK_4 derived, listed in Table I.

Stability of the drug radicals in the absence of oxygen

As with tirapazamine, pulse radiolysis generation of the mono-*N*-oxide radicals was followed by radical decay in anoxia over tens of milliseconds. The radical stability was dependent on radiation dose, i.e. on radical concentration since the decay was found to be second order and the first half-life of the radicals inversely proportional to radical concentration:

$$\text{anoxic half-life} = [2k_3(\text{radical})]^{-1} \quad (7)$$

The rates of decay $[-d(\text{radical})/dt = 2k_3(\text{radical})^2]$ were dependent on pH for RB91724, $2k_3$ decreasing from 1×10^8 to 1.2×10^7 dm^3 mol^{-1} s^{-1} from pH 5 to pH 9.5 [*cf* the pH-dependent lifetime in anoxia of the tirapazamine radical (Laderoute *et al.*, 1988)]. The other RB compounds investigated showed less dependence of $2k_3$ on pH over this range. Estimates of the decay rate constants close to physiological pH are given in Table I.

Rates of reaction of the drug radicals with oxygen at pH 7.4

Pulse radiolysis of solutions containing formate (0.1 mol dm^{-3}), *N*-oxide (0.1–0.5 mmol dm^{-3}) and nitrous oxide–oxygen mixtures (0.5–10% O_2 v/v) at pH ~7.4 gave transient absorptions of the drug radicals decaying exponentially on timescales 1–2 orders of magnitude faster than in the absence of oxygen. The rate of decay was proportional to oxygen concentration [*cf* tirapazamine (Laderoute *et al.*, 1988; Wardman *et al.*, 1994)], and estimates of the rate constant for reaction of drug radical with oxygen, k_2 were obtained (Table I). At the higher oxygen concentrations, some superoxide radicals would be formed from direct electron transfer from $CO_2^{\bullet-}$ to O_2; this did not appear to influence the lifetime of drug radicals at the low total radical concentrations used.

Reactions of viologen radicals with N*-oxides, and drug radicals with viologens*

Radicals ($V^{\bullet+}$) from one electron reduction of viologens (V^{2+}) (e.g. 1,1′-dimethyl-4,4′-bipyridinium dichloride, MV^{2+}) are stable in the absence of oxygen and absorb intensely at 400 and 600 nm where the *N*-oxide radicals do not absorb (Michaelis and Hill, 1933). They are often used as redox indicators to measure reduction potentials of drug–drug radical one electron couples (Wardman, 1989, 1991), by measuring the equilibrium constant of the electron transfer equilibrium:

$$\text{drug radical} + V^{2+} \rightleftharpoons \text{drug} + V^{\bullet+} \quad (8)$$

Pulse radiolysis of solutions containing formate (0.1 mol dm^{-3}), *N*-oxide (~0.7 mmol dm^{-3}) and MV^{2+} (~25 μmol dm^{-3}) showed the absorption of $MV^{\bullet+}$ at 600 nm grew exponentially in ~200 μs. Variation of the MV^{2+} concentration showed the rate to be first order in $[MV^{2+}]$ and this is ascribed to the forward reaction in equilibrium (8). An estimate of k_{8f} is given in Table I. Pulse radiolysis of solutions containing formate (0.1 mol dm^{-3}), MV^{2+} (5 mmol dm^{-3}), *N*-oxide (~0.2 mmol dm^{-3}), nitrous oxide (N_2O), pH 7.4 showed that the absorption at 600 nm of $MV^{\bullet+}$ decayed exponentially at a rate proportional to drug concentration over ~1 s. This is ascribed to the reverse reaction of equilibrium (8), yielding values for k_{8r} given in Table I. The equilibrium constant, K_8 is calculated as k_{8f}/k_{8r} (Table I). There was no clear reaction between $MV^{\bullet+}$ and RB90740, but reaction of $MV^{\bullet+}$ with tirapazamine was similarly observed (Table I). These experiments utilised pH > pK_4 so that >70% of the drug radicals were unprotonated (Table I).

Table I Properties of radicals derived from *N*-oxides

Compound	pK_5	pK_4	$10^{-7}(2k_3)^a$	$10^{-7}k_2{}^a$	$10^{-8}k_{8f}{}^a$	$10^{-3}k_{8r}{}^a$	10^{-4} K_8
Tirapazamine	12.5	6.0	7.8	0.80		58	
SR4482		~5.0	~2	~0.1			
SR4718			4.5	0.18			
SR4895			3.9	0.20			
SR4452	8.0		2.1	0.13			
RB91724	5.6	7.4	1.4	16	3.2	1.8	1.8
RB90740	5.0	6.2	11	32	14	ND[b]	
RB93918	6.0	5.5	16	18	6.3	1.0	63

[a] dm^3 mol^{-1} s^{-1}. [b] Not determined.

Benzyl viologen (BV^{2+}) is 0.076 V more electron-affinic than MV^{2+} (Wardman, 1991), and similar experiments with the three mono *N*-oxides yielded estimates of k_{8f} with BV^{2+} which were $\approx$2 to 4 times higher than those for MV^{2+}. The reverse rate constant, k_{8r} was too low to be determined with BV^{2+}.

Reduction of N-*oxides in the presence of hydrogen atom donors*

The solutes used in radiation chemistry to 'convert' oxidising hydroxyl radicals to one electron reductants such as $CO_2^{\bullet-}$ or alcohol radicals are hydrogen donors: hydroxyl abstracts hydrogen to produce the reducing radical. The radical yield in radiation chemistry is known or measurable precisely, and it was shown in earlier work (Laderoute *et al.*, 1988) that at pH 5.8–6.8, more tirapazamine was reduced than the yield of $CO_2^{\bullet-}$ in the presence of the H donor, formate. This was explained by the drug radical abstracting H from formate (H donor, RH) to yield another reducing radical, R•, in turn generating another tirapazamine radical in a short chain reaction terminated by radical–radical reaction (3):

Initiation: $^{\bullet}OH + RH \rightarrow R^{\bullet}$ (9)

Propagation: $R^{\bullet}$ + drug → R′ + drug radical (10)

drug radical + RH → drug reduction product + $R^{\bullet}$ (11)

Termination: 2 drug radicals → reduction product(s) (3)

(The reducing radicals R• react too rapidly with the drug for termination by radical–radical reactions involving R• to be important.) These earlier experiments involved measuring the amount of $CO_2^{\bullet-}$ needed to completely reduce tirapazamine, with the complication that the 1-oxide reduction product (SR4317) is also highly reactive towards the reducing radicals (data not shown), and further reduction products are also produced.

We have extended these studies, involving measuring loss of drug and product formation at only a few per cent conversion of drug so that side-reactions are minimised, varying pH, dose-rate and concentration of H donor over a wide range, using formate, 2-propanol and deoxyribose as H donors and comparing tirapazamine with the mono *N*-oxides. Because a mixture of products was observed in all cases, the most useful data are the loss of drug as a function of radical input; thus one drug molecule would be removed by two reducing radicals R• if reduction only involved the two electron product, e.g. the 1-oxide (SR4317) in the case of tirapazamine. Any higher value than 0.5 for the drug lost/radical input reflects the occurrence of the chain reaction hypothesised above. An example of the results obtained with tirapazamine and deoxyribose has been reported elsewhere (Wardman *et al.*, 1995), and Table II summarises the main results. Precise values varied with drug, H donor and its concentration, dose rate (radical generation rate) but especially pH. Full details will be reported elsewhere, but in every case the reduction stoichiometry was approximately 0.5 drug lost/radical input at high pH, and approximately 2–5 molecules drug lost/radical input at pH 5 or less. The approximate points of inflexion ('mid-point') on the stoichiometry/pH profile are also shown in Table II.

Table II Chain reduction of *N*-oxides: molecules drug lost per radical input (dose rate 5–7 Gy min^{-1})

Compound	*H donor*[a]	*pH* ~ 5	*pH* ~ 10	*'pK*$_a$[b]
Tirapazamine	HCO_2^-	4.6	0.8	~7.6
Tirapazamine	2-PrOH	2.9	0.7	~7.3
Tirapazamine	dR[c]	3.0	0.4	~7.8
RB91724	HCO_2^-	2.9	0.6	~6.1
RB93918	HCO_2^-	1.9	0.6	~7.5

[a]0.1 mol dm^{-3}. [b]Point of inflexion in the curve of drug lost *vs* pH. [c]2-Deoxy-D-ribose.

Reduction of N-*oxide drugs by deoxyribose radicals*

Pulse radiolysis of N_2O-saturated solutions containing deoxyribose (50 mmol dm^{-3}) and RB91724 (0.1 mmol dm^{-3}), pH 5 produced absorption changes identical to those obtained on reduction by $CO_2^{\bullet-}$ (formate system), except that the yield of drug radicals was reduced to close to 50%. The absorption decayed rapidly in the presence of oxygen at a similar rate to that observed in the formate system.

Discussion

We have shown that the mono-*N*-oxide radicals react with oxygen, reaction (2), $\approx$ 10–40 times faster than does the tirapazamine radical. The anoxic stabilities of the radicals with respect to disproportionation, reaction (3), do not differ greatly but are pH-dependent to differing degrees. Both classes of *N*-oxide radicals undergo prototropic equilibria, equation (4), with pK_a values in a broadly similar region, but the site of protonation of the radical-anion has not been identified. [Pulse radiolysis with conductimetric detection (data not shown) indicates the tirapazamine radical to be anionic at pH >> pK_4.] The ground state pK_a values (pK_5) probably refer to ionisation of the -NHR proton in the 3-substituent in tirapazamine and SR4452, but to an unidentified protonation site in the RB series of compounds. In neither class is it likely that pK_4 and pK_5 refer to the same prototropic site in radical and ground state.

Some progress has been made in estimating thermodynamically reversible reduction potentials for the drug radical–one electron couples in water. [Previous electrochemical measurements (Zeman *et al.*, 1989; Minchinton *et al.*, 1992; Naylor *et al.*, 1993; Tocher and Edwards, 1994) reflect different parameters and cannot be compared directly.] From K_8 it is easily calculated (Wardman, 1989) that the one-electron reduction potentials at pH 7 are −0.70 and −0.79 V *vs* NHE for RB91724 and RB93918 respectively. These compounds are significantly less electron-affinic than misonidazole (− 0.40 V) and metronidazole (− 0.50 V), and their activation by the common reductases is surprising in view of the relationships between reduction rate and potential (Wardman *et al.*, 1995). We have recently similarly estimated the analogous reduction potential to be ~ −0.45 V for tirapazamine (data not shown). These values are reflected in the relative rates of electron transfer of the *N*-oxide drug radicals to oxygen.

The chain reaction in the radiolytic reduction of tirapazamine and formate (Laderoute *et al.*, 1988) has been extended to other *N*-oxide radicals and H donors, including deoxyribose. Quantitative analysis of the data is complicated by the observation with tirapazamine and one of the mono-*N*-oxides that the pH-dependence, while strongly suggesting the protonated radical is much more reactive the the radical-anion, shows changes over 1–2 pH units higher than the radical pK_a (pK_4). Neglecting this gap in our understanding, the reaction sequence (9),(10),(11),(3) (see above) can easily be analysed using the steady-state approximation to estimate the magnitude of the H abstraction rate constant, k_{11}. The tirapazamine/formate data suggested k_{11} ~200 dm^3 mol^{-1} s^{-1}, and overall the range with the different *N*-oxides studied to date and the differing H donors suggest k_{11} is generally ~10^2–10^3 dm^3 mol^{-1} s^{-1}. We plan to extend these studies to provide a quantitative scale of H abstraction rates with the different drugs.

The high reactivity with oxygen of the radicals obtained on one electron reduction of all the *N*-oxides studied strongly supports the view that the selective toxicity of both classes of compounds towards hypoxic cells can be ascribed to the

'futile cycling' of the drug radicals in the presence of oxygen (Laderoute *et al.*, 1988). This is likely to be the case if activation involves e.g. cytochrome *P*-450 reductase, but not with DT-diaphorase (an enzyme not reducing via radical intermediates), a factor to be considered in enzyme-directed bioreductive drug development (Workman and Stratford, 1993). Oxygen dependences of cytotoxicity of tirapazamine (Koch, 1993) and RB90740 (Naylor, 1994) have been reported. They are qualitatively different, but oxygen protects against cytotoxicity with the latter compound at considerably lower concentrations than with tirapazamine. This is in line with the ~40-fold higher rate constant for reaction of the drug radical with oxygen of RB90740 than the corresponding value for the tirapazamine radical. Too high a rate constant could prevent radioresistant hypoxic (but not anoxic) cells being killed by the cytotoxin; RB90740 has been found to have poor *in vivo* efficacy, attributed to such an unfavourable oxygen dependence (Naylor, 1994).

We have discussed elsewhere (Wardman *et al.*, 1995) simple models for the competing reactions which might occur in hypoxic cells. If these *N*-oxides act by the radical abstracting hydrogen e.g. from a sugar residue in DNA to initiate a strand break (Laderoute *et al.*, 1988), then we can easily derive a scheme in which drug activation to a radical (1) is followed by a reaction leading to toxicity involving H abstraction, (11) (the protonated drug radical being the most reactive form). Reaction (11) is in competition with radical reaction with oxygen (2) and, possibly, radical–radical disproportionation (3), both of which are deactivation pathways. If reaction (3) is not involved because of the extremely low steady-state radical concentrations which would occur in all except anoxic cells, then the model reduces to the simplest case of two competing first-order pathways, and the oxygen concentration at which cytotoxicity will be midway between the anoxic and highly oxic levels will be when the two pathways are balanced, i.e. when $k_2[O_2] = k_{11}[RH.]$ [Pathway (11) includes all H-abstraction reactions of the drug radical, not just the critical reaction leading to cytotoxicity.] This only applies to homogeneous kinetics, and cannot simply be extrapolated to the cellular environment, but e.g. if $k_2 \approx 10^7$ to 10^8 dm^3 mol^{-1} s^{-1}, $k_{11} \approx 10^2$ to 10^3 dm^3 mol^{-1} s^{-1} and [RH] = 1 mol dm^{-3}, then [O_2] (the '*K*-value') would be ≈1 to 100 μmol dm^{-3} ($pO_2 \approx 0.8$ to 80 mmHg, 37°C). The unusual concentration dependence of tirapazamine cytotoxicity (Koch, 1993) cannot be explained by this simple model, although we note the overall variation is largely encompassed by the range of rate constants/H donor concentration described above.

In earlier discussion of the implications of the radiolytic chain reduction (Wardman *et al.*, 1995) we considered the possibility that a second stage after H abstraction by the radical was the generation of another drug radical by a modified, reducing DNA radical which might remain after strand breakage. This second drug radical might induce damage close to the first lesion by the chain mechanism of our model, thus possibly explaining the 'clustered' damage which has been reported, rather akin to that observed with high LET radiation (more difficult to repair than simple single strand breaks) (Wang *et al.*, 1992). With the limitations of homogeneous kinetics, we can consider the competition between diffusion of the second radical away from the site of generation and its reaction close to the original site of damage. The mean diffusion distance of a radical will be $(2Dt)^{1/2}$, where D is the diffusion coefficient and t the mean radical lifetime. Taking a reasonable value of D in the cytoplasm, we obtain a diffusion distance of $17(t^{1/2})$ μm, if t is in seconds (Wardman *et al.*, 1995). If $k_{11}[RH]$ [the product of radical H abstraction rate and effective target concentration ('lethal' and 'non-lethal' pathways summed)] is $\approx 10^2$ to 10^3 s^{-1}, then the radical might live for ≈1 to 10 ms in anoxia and therefore diffuse ≈0.5 to 2μm (diffusion will be less with O_2 present). This considerable distance casts doubt on our original hypothesis (Wardman *et al.*, 1995) that a single drug radical could lead to damage amplification in the way we suggested. However, direct amplification by the damage (DNA radical centre), necessarily remaining after the strand break, reacting with drug to modify the damage seems more likely [*cf* radical addition/elimination reactions of radiosensitisers (Wardman, 1993)].

Further comparison of the chemical properties of the drugs with the biological properties is premature until our chemical models are refined and extended, but measurements of the oxygen dependence of cytotoxicity for more analogues would be very useful. We suggest for the present that the three most important chemical properties controlling aromatic *N*-oxide drug efficacy are reduction potential, rate constant for the drug radical reacting with oxygen, and radical pK_a.

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