Radicals from one-electron reduction of nitro compounds, aromatic N-oxides and quinones: the kinetic basis for hypoxia-selective, bioreductive drugs

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Abstract

Drugs based on nitroarene, aromatic N-oxide or quinone structures are frequently reduced by cellular reductases to toxic products. Reduction often involves free radicals as intermediates which react rapidly with oxygen to form superoxide radicals, inhibiting drug reduction. The elevation of cellular oxidative stress accompanying oxygen inhibition of reduction is generally less damaging than drug reduction to toxic products, so the drugs offer selective toxicity to hypoxic cells. Since such cells are resistant to radiotherapy, these bioreductive drugs offer potential in tumour therapy. The basis for the selectivity of action entails kinetic competition involving the contesting reaction pathways. The reduction potential of the drug, radical pKₐ and nature of radical/radical decay kinetics all influence drug activity and selectivity, including the range of oxygen tensions over which the drug offers selective toxicity. These properties may be quantified using generation of radicals by pulse radiolysis, presenting a physico-chemical basis for rational drug design.

Introduction

Nitro compounds, aromatic N-oxides and quinones share the common property of being readily reduced by biologically relevant reductants. Paradoxically, reduction of the compounds (S) can stimulate cellular oxidative stress in

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an aerobic environment where reduction involves the generation of radicals (SH'/S') as obligate intermediates (eqn. 1) or where radicals can be formed by reaction of oxidant (S) with the reduced compound (SH₂) (eqn. 2). This is because superoxide radicals (O₂⁻) can be formed (eqn. 3), which in turn can produce hydrogen peroxide (eqn. 4) and hydroxyl radicals (·OH) directly via Fenton chemistry (eqn. 5) or through analogous chemistry involving hypochlorous acid:

\[
\begin{align*}
S + \text{reductant} \pm \text{enzyme} & \rightarrow S^- (+H^+ \rightleftharpoons SH') \\
S + SH₂ & \rightleftharpoons 2SH' \rightleftharpoons 2S^- + 2H^+ \\
S^- + O₂ & \rightleftharpoons S + O₂^- \\
2O₂^- + 2H^+ & \rightarrow H₂O₂ + O₂ \\
H₂O₂ + \text{reduced metal} & \rightarrow \cdotOH + OH^- + \text{oxidized metal}
\end{align*}
\]

In the absence of oxygen, reactions (3)–(5) will not be stimulated by the presence of the oxidant S; however, damaging reactions of radicals or other reduction intermediates may occur with biological targets:

\[
\begin{align*}
\text{SH'/S'}^- + \text{target} & \rightarrow \text{damage} \\
\text{SH}_2 + \text{target} & \rightarrow \text{damage}
\end{align*}
\]

The extent of these competing pathways will obviously depend not only on oxygen tension but also on levels of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, which can frequently prevent reactions (4) and (5) leading to the formation of highly reactive hydroxyl radicals by removing O₂⁻ and/or H₂O₂. The outcome of drug reduction in aerobic or hypoxic environments thus depends on which is the greater of two evils: stimulating cellular oxidative stress or forming reactive drug-reduction intermediates. This competition forms the basis for the rational design of hypoxia-selective bioreductive drugs, as outlined in Fig. 1. When drug reduction leads to more toxic pathways than superoxide formation, either because of low oxygen tension or chemical reactivity, then selective toxicity towards hypoxic cells or organisms will occur. Reactions (1) and (3) together constitute a redox cycling or futile cycling role for the drug, in that it can stimulate cellular oxidative stress without the drug being consumed in the process [1].

Although the prototypical bioreductive drug, metronidazole (Flagyl), is widely used in medicine, there is much current interest in targeting hypoxic cells in tumours. This arises in part from the radioresistance of hypoxic cells. Up to three times higher radiation dose may be required to kill hypoxic cells compared with well-oxygenated cells; if non-cycling, such cells may also be resistant to some chemotherapeutic drugs [2].

This article outlines the chemical kinetic basis for drug selectivity of bioreductive drugs, the majority of which involve nitroarene, aromatic N-oxide or quinone structures. The properties of radical intermediates in drug action are discussed, stressing the similarities between the three groups of compounds and noting key differences where they occur. Overviews of some of the kinetic aspects
of radical reactions involving nitroarenes [3] or quinones [4] were presented some time ago. More detailed reviews of the radical chemistry and mechanisms of toxicity of quinones, in particular, have been published [5–7] and the applications of EPR spectroscopy to detect redox cycling radical intermediates have been described [1,8]. Damage to DNA by redox-cycling drugs has been discussed [9]. Recently, emphasis on the design of bioreductive drugs has focused on enzyme-directed drug development [10] and N-oxides have attracted increasing attention [11–13]. However, the nitroarene moiety remains an important basis for bioreductive drugs [14–16].

We do not attempt to present here a comprehensive review of bioreductive drug mechanisms. Rather, we seek simply to present a framework for the logical discussion of chemical pathways based on known or accessible kinetic and thermodynamic information.

**Prototypical compounds**

Metronidazole (Fig. 2; 1) is probably the nitroarene most widely used in medicine. It is closely related to the hypoxic cell radiosensitizer, misonidazole (2) [2,17]. The dinitrobenzene, CB 1954 (3), has been considered recently as a candidate for antibody-directed enzyme-prodrug therapy [18]. The related dinitrobenzene (4) has higher specificity for hypoxic cells than CB 1954 [19]. Nitrofurans such as nitrofurantoin (5) are used as urinary antibacterials. Nitroquinolines such as 6 are currently being studied in detail as hypoxic cell cytotoxins [20,21].

2-Methyl-1,4-naphthoquinone (menadione, 7) is an analogue of vitamin K, but the 2,3-dimethoxy analogue differs in its biological reactivity because the rapid
Radicals from one-electron reduction

Fig. 2. Typical bioreductive drugs under experimental or clinical evaluation, based on nitroarene, quinone or aromatic N-oxide moieties.

congjugation/reduction characteristic of menadione and glutathione is blocked by the two methoxy substituents [6,22,23]. Mitomycin c (8) might be viewed as a model quinonoid bioreductive drug, although its oxic/hypoxic differential toxicity is smaller than that of some newer drugs [24,25]. Recent interest in related structures has focused on EO9 (9). Hydroxy substituents capable of hydrogen bonding to the quinone oxygens is a feature of the chemotherapeutic drug, doxorubicin (adriamycin, 10). Whether the latter is a bioreductive drug in the
conventional sense is unlikely; although this possibility remains a matter of some
dispute [26], redox cycling to stimulate oxidative stress may be associated with the
dose-limiting cardiotoxicity [9,27,28]. Substituted benzoquinones of interest
include diazaquone (AZQ; 11) and recently reported 1,2-benzoquinone analogues
such as 12 [29].

Quinoxaline-di-N-oxides such as quinoxin (13) were used as poultry feed
additives many years ago and radical intermediates were implicated in their mode
of action [30]. Most current interest in aromatic N-oxides focuses on the
benzotriazine, tirapazamine (SR 4233, 14), which is selectively toxic towards
hypoxic mammalian cells [31–33]. Although the mono-N-oxide analogue of
tirapazamine, SR 4317 (the 1-oxide), has low toxicity, some mono-N-oxides,
including bifunctional compounds such as 15 (with both N-oxide and nitroarene
moieties), are of current interest as hypoxia-specific cytoxins [13]. Aliphatic
N-oxides include pro-drugs of DNA-binding agents, activated by reduction, but
their mode of action does not appear to involve radical intermediates [34].

Mechanisms of bioreductive drug cytotoxicity

This article concentrates on the kinetics of radical reactions, and a compre-
hensive survey of mechanisms of cytotoxicity is inappropriate here. Reviews of
radical mechanisms, particularly of quinones, were noted in the Introduction. In
addition, brief overviews have been presented of some mechanistic aspects in
relation to cancer therapy [35] and trichomoniasis [36]. Three distinct pathways to
cytotoxicity can be envisaged, and an outline will help set the scene for a discus-
sion of the kinetics of critical free-radical reactions.

Product from disproportionation of drug radicals may be toxic

Drug radicals are generally unstable at physiological pH (see below). Radical–
radical reactions in many instances involve disproportionation. Nitroarene radicals
provide a typical example; quinone radical disproportionation is the reverse of eqn.
(2), but there have been no reports of radical generation on mixing the di-N-
oxide, tirapazamine (Fig. 2; 14), with the two-electron reduced product, the
mono-N-oxide. Nitroarene radicals yield a nitrosoarene (RNO) upon dispropor-
tionation (eqn. 8), which is generally reduced further to a hydroxylamine
(RNHOH) by e.g. glutathione (eqn. 9) or ascorbate, or nitro radicals:

$$2\text{RNO}_2^- + 2\text{H}^+ \rightarrow \text{RNO} + \text{RNO}_2^- + \text{H}_2\text{O}$$ (8)

$$\text{RNO} + \text{GSH} \rightleftharpoons \text{RN(OH)SG} + \text{GSH} \rightarrow \text{RNHOH} + \text{GSSG} \text{(or } \rightarrow \text{RNHSOG})$$ (9)

Reaction with GSH (eqn. 9) is quite complex, as indicated above [37–40], but the
half-lives of reaction with, for example, 2 mmol/dm$^3$ GSH and representative
nitrosobenzenes are in the range 4–70 ms [38]. A thorough kinetic study has been
reported [40]. Free glutathione will be in competition with protein thiols for
reaction with nitrosoarenes. 2-Nitrosoimidazoles [41–47] and 5-nitrosoimidazoles
[48,49] have been prepared, and their cytotoxicity and mutagenicity have been
assessed. Hydroxylamines derived from 2-nitroimidazoles are unstable but
reactivity of these and other nitroarene reduction products towards nucleophilic bases on DNA (e.g. guanine) has been demonstrated in model systems [44,50–61].

**Reduction activates potentially toxic functional groups**

Since radical intermediates are not necessarily involved in this route to toxicity, only very brief mention is appropriate here. Nitroarene or quinone moieties effectively deactivate alkylating substituents, and reduction restores the reactivity. Prototypical compounds are CB 1954 (Fig. 2; 3) [18], mitomycin c (8) [24,62,63] and diazaquone (11) [5]. Another example, not shown, would be the misonidazole (2) derivative in which the side-chain terminal methoxy function is replaced by aziridine, RSU 1069, or pro-drugs of this compound [14]. This group of compounds especially is the focus of enzyme-directed bioreductive drug research [10,35], since two-electron reductants such as DT-diaphorase can activate many such drugs, particularly quinones.

Reduction by one-electron, i.e. to the radical, is probably sufficient to activate the alkylating substituents, but whether the radical reacts as an alkylator depends on competing kinetics of radical–radical reactions and alkylation. Quantitative information is lacking.

**The drug radical reacts directly with a cellular target**

Although nitro radical reactivity towards DNA bases has been deduced from electrochemical measurements and the nature of the damage [51,64–68], quantitative rate data are unavailable, and it remains conjecture whether radical–DNA reactions can occur before other reactions of the radicals in cellular systems. Electrochemical studies of the DNA-damaging reactions of tirapazamine (Fig. 2; 14) show a pH effect [69] which is consistent with the model for DNA damage via the protonated radical which is discussed below. The main supporting evidence for this mechanism is the lack of cytotoxicity associated with the stable 2- or 4-electron reduction products [70].

**Factors controlling bioreductive drug activation**

**Reduction rate: obligate one-electron reduction**

Reaction (1) above is a general expression of one-electron reduction. A representative simple chemical model for enzyme-catalysed reduction is reaction with dihydroflavins, FMNH$_2$, in the absence of oxygen:

$$ S + \text{FMNH}_2 \rightarrow \text{SH}_2 + \text{FMN} $$

(10)

In the case of nitro compounds (RNO$_2$), the reaction was found [71] to follow stoichiometry corresponding to reduction to the hydroxylamine, RNHOH:

$$ \text{RNO}_2 + 2\text{FMNH}_2 \rightarrow \text{RNHOH} + 2\text{FMN} $$

(11)

but the initial rate law for reduction of several nitroimidazoles was approximately $k_{11}[\text{RNO}_2][\text{FMNH}_2]$ and Fig. 3 shows that the rate constant for reduction [71] increased with the reduction potential for adding a single electron [72]. Reduction
of nitroacridines by FMNH₂ showed a virtually identical redox dependence and absolute reactivity [73], as shown in Fig. 3.

Xanthine oxidase normally catalyses the reduction of oxygen to superoxide with xanthine or hypoxanthine as cofactor, but, in the absence of oxygen, nitro compounds or other bioreductive drugs act as alternative electron acceptors. Clarke et al. [74] measured the kinetics of reduction of nitroimidazoles by xanthine/xanthine oxidase and estimated the initial rates at a concentration probably rather less than the Michaelis $K_m$ value. The redox dependence of this flavoenzyme was quite similar to that found with free flavins; the data are shown in Fig. 3.

Probably the most important reductase in mammalian tissues reducing bioreductive drugs to radical intermediates is NADPH-cytochrome P-450 reductase, and the enzyme kinetics for oxygen consumption, reflecting reactions (1), (3) and (4) with (1) rate-limiting, was found to have a similar redox dependence to the xanthine oxidase system [75]. The authors estimated the Michaelis parameters $V_{max}/K_m$; this ratio, in the units used in the report [75], is plotted in Fig. 3. It approximates to the effective rate constant at substrate concentrations much less than $K_m$. In the same study [75], reduction by ferredoxin:NADP⁺ oxidoreductase was measured, and the data in Fig. 3 demonstrate a similar redox dependence of reduction rate on catalysis by cytochrome P-450 reductase and xanthine oxidase. A later study [76] quantified radical formation using cytochrome c reduction as indicator rather than oxygen consumption, and the broken line in Fig. 3 shows the redox dependence for reduction by cytochrome P-450 reductase for compounds with reduction potential $<-0.15$ V. Although the redox dependence was significantly lower than in the earlier studies with this and other

Fig. 3. Redox dependences of relative rates of reduction of nitroarenes (logarithmic scale). ○, FMNH₂ + nitroimidazoles; ●, FMNH₂ + nitroacridines; □, xanthine/xanthine oxidase + nitroimidazoles; ■, ferredoxin: NADP⁺ oxidoreductase; Δ, cytochrome P-450 reductase; broken line, cytochrome P-450 reductase + nitroarenes and quinones (see text for sources of data).
enzymes, an important result was that reduction of quinones and nitro compounds followed similar kinetics, depending only on reduction potential. (A recent study [77] measured $K_m = 3 \mu$mol/dm$^3$ for 2-methylmethoxy-1,4-naphthoquinone and cytochrome P-450 reductase. Thus the use of a single concentration of 50 $\mu$mol/dm$^3$ in the work of Butler and Hoey [76], higher than the probable $K_m$ for some of the compounds included, may not have been appropriate for the redox analysis. The same criticism may hold partially true for the earlier study with xanthine oxidase [74] for the most electron-affinic compounds.)

None of these investigations measured directly the rate of formation of radical-anions, but from extensive studies using EPR detection of steady-state concentrations of free radicals, it seems likely that the reductases described above (and free, reduced flavins) reduce nitro compounds [78], quinones [5] and aromatic N-oxides [79] by generating radical-anions as obligate intermediates. Radical formation rates are evidently strongly dependent on reduction potential. Cellular reduction, including by mammalian cells, has a redox dependence close to those shown in Fig. 3 [80], as does cytotoxicity towards anoxic mammalian cells [81].

**Radical formation via two-electron reduction or thiol conjugation**

Eqn. (2) implies that if a drug $S$ is reduced by two electrons to $SH_2$, then radicals $S^-/SH^-$ can still be formed if equilibrium (2) is favourable. In practice, such radical formation has been characterized only for quinones, and does not seem to occur with nitroarenes or aromatic N-oxides at physiological pH. With quinones, equilibrium (2) is pH-sensitive; thus for 2,3,5,6-tetramethyl-1,4-benzoquinone (dudoquinone), the semiquinone formation constant, $K_f$ increases from $\sim 10^{-14}$ at pH $<4$ to $\sim 1$ at pH $\sim 14$ [82]:

$$K_f = K_a = ([Q^-]_{tot})^2/([Q]_{tot} [QH_2]_{tot})$$

where the subscripts denote total concentrations of the species, summing all prototropic forms. At high pH, the duroquinone radical-anion is produced in high concentration, and is stable, on mixing the quinone and hydroquinone [83]. Factors influencing the magnitude of $K_f$ include the presence of OH substituents where the proton can hydrogen bond to the quinone oxygen, thus stabilizing the radical. This feature is seen in doxorubicin (Fig. 2; 10), and a particular feature of this drug is that its semiquinone formation constant at pH 7 is about nine orders of magnitude higher than that for duroquinone [84]. Thus doxorubicin reduction, even by enzymes not involving radical intermediates, can potentially stimulate redox cycling via reactions (2) and (3). This may be the basis for the drug's cardiotoxicity [9]. Reducing redox cycling by intentional manipulation of $K_f$, by varying the substituents to control the p$K_a$ values which influence this parameter, does not seem to have been attempted.

Radical formation via the reduction product $SH_2$ will only occur by this route if $SH_2$ is sufficiently stable to allow significant concentrations to build up to permit the establishment of equilibrium (2). This may not be the case, since with the indoloquinone EO9 (Fig. 2; 9) the toxicity linked to the obligate two-electron reductant, DT-diaphorase, is not oxygen-sensitive. Indeed, there is an inverse correlation between the levels of DT-diaphorase and hypoxic sensitivity to EO9
This may imply that reduction activates the aziridine alkylating substituent and this functional group is then so reactive that free hydroquinone cannot build up to generate radicals. This may also explain the fairly low hypoxic/oxic selectivity of mitomycin c (Fig. 2; 8) [24]. Enzyme-directed bioreductive drug therapy [10] and the role of DT-diaphorase in quinone activation [24,25,63,77] are complex topics outside the scope of this article.

The quinone moiety generally represents a highly activated \( \alpha, \beta \)-unsaturated ketone and is particularly sensitive to Michael addition. Thus in menadione (Fig. 2; 7), the unprotected (e.g. by substitution) 3-position is liable to attack by thiolate nucleophiles [86]. The conjugate thioether is a hydroquinone and thus can lead to radical formation via equilibrium (2) [or eqn. (14)]; thiol conjugation is equivalent to reduction:

\[
\begin{align*}
Q + GSH (+H^+) & \rightarrow Q(SG)H_2 \\
Q + Q(SG)H_2 & \rightleftharpoons QH' + Q(SG)H'^+ \\
Q + q(SG)H'^+ & \rightleftharpoons QH'^+ + Q(SG)
\end{align*}
\]

The reactions with glutathione are rapid [22,87] and even the fastest examples are accelerated by glutathione S-transferases [88]. Thioether formation has only a small effect on the reduction potential of menadione [89], so the thiol conjugate, when autoxidized, will redox cycle efficiently. Semiquinone radicals may also oxidize thiols to produce thyl radicals [77], and oxidized glutathione is then produced [77,90]. Glutathione is involved in reductive activation of mitomycin c, involving reactions other than reduction of the quinone (eqn. 13) [91,92].

Direct detection of quinone radicals formed on mixing glutathione and menadione proved unequivocally the potential importance of radical generation via reactions (13)–(15) [93,94]. Which radical predominates at steady state depends on the extent of reaction, on the redox equilibrium (eqn. 15), and on the decay kinetics of the radicals. Pulse radiolysis data [89] yield an estimate of \( K_{15} \approx 0.6 \).

**Factors controlling the rate of reaction of the drug radicals with oxygen**

**Energetics**

Reaction (3) is formally an electron-transfer equilibrium, with a free-energy change, \( \Delta G \), given by:

\[
\Delta G_3 = -nF \Delta E_3 = -RT \ln K_3
\]

where \( n = 1 \), \( F \) is the Faraday constant, \( R \) the gas constant, \( T \) the absolute temperature and \( K_3 \) the equilibrium constant; \( \Delta E_3 \) is defined by:

\[
\Delta E_3 = E_{mi}(O_2/O_2^-) - E_{mi}(S/S^-)
\]

where \( E_{mi} \) represents the mid-point potentials of the one-electron couples at pH i. The latter are most reliably determined by pulse radiolysis measurements of electron-transfer equilibria before the partners in the equilibrium can decay [72]. These potentials can often be predicted if those of analogues are known [95]. For
comparison of electron affinities we use a (non) standard state for O$_2$ of 1 mol·dm$^{-3}$, when $E_{m7}(O_2^-) = -0.18$ V versus NHE [96]. Then from eqns. (16) and (17):

$$\log K_3 \approx -3.04 - 16.9E_{m7}(S/S'^-$$

(18)

if potentials are in volts.

The higher the value of $E_{m7}(S/S'^-)$, the lower the energy ‘driving’ electron transfer to oxygen, and from Marcus theory, the slower electron transfer is expected to be [97]. Fig. 4 summarizes some rate data for reactions of quinone and nitro radicals with oxygen, which show that the general behaviour is as expected. The quinone data have been presented previously [4], and extend the earlier ‘Marcus type’ correlation [98]. For quinone radicals with low values of $E_{m7}(S/S'^-)$, the rate constants are not much lower than the diffusion-controlled limit. In contrast, for similar energetics, nitro radicals react with oxygen around two orders of magnitude more slowly than quinone radicals. The data plotted extend the initial study [99] and include unpublished measurements (P. Wardman and E. D. Clarke, unpublished work).

For both nitro and quinone radicals, over a wide range, the higher the reduction potential, the slower the electron transfer to oxygen. This redox relationship is therefore of opposite direction to drug activation to form free radicals, as plotted in Fig. 3. Also shown in Fig. 4 is the value for the rate constant for reaction of the radical from the N-oxide, tirapazamine, with oxygen [100,101]. It is very similar to the value for the metronidazole radical [99].

**Kinetics of electron exchange**

The main point of Fig. 4 merits reinforcement: reduction potentials influence, but do not in themselves define, electron transfer rates. Equally important are the ‘zero energy’ electron-exchange rates [97,98]. Nitro compounds are kinetically
sluggish in electron transfer reactions generally, and it may well be that reaction of nitro radicals with oxygen involves an intermediate adduct, just as nitro compounds themselves tend to form adducts with reducing radicals [102]. Hence it is worth stressing that the kinetics of reaction (3) may vary over a wide range for different bioreductive drugs, apart from the influence of reduction potential. Fortunately, these reactions are easily characterized by pulse radiolysis.

Factors controlling the stability of radicals in anoxia: radical–radical reactions

Prototropic equilibria of radicals
Radical disproportionation to form a two-electron reduction product is the reverse of eqn. (2) [cf. eqn. (8)]:

$$2S^- (or \text{SH}^\cdot) \rightarrow \text{SH}_2 + S \quad (19)$$

and as described above, may represent an important stage in a pathway to toxic reduction products (as with nitroarenes) or a protective step, reducing the lifetime of potentially toxic radicals by forming a non-toxic product, as with tirapazamine. In either case the rates of this radical–radical reaction are important since radical disproportionation, eqn. (19), competes with reaction with oxygen, eqn. (3), or with a target, eqn. (6).

Key factors controlling radical stability in anoxia are prototropic equilibria of radicals and pH. Fig. 5 illustrates both factors with the well-known superoxide radical [103], the tirapazamine radical [100] and the metronidazole radical [104]. (Note the rate constant is defined by the formalism: $-d[R]/dt = 2k[R]^2$, where [R] is the sum of the concentrations of all the prototropic forms of the radical.)

![Graph showing rate constants for radical–radical reactions as a function of pH](image)

**Fig. 5.** Rate constants (logarithmic scale) for radical–radical reactions as a function of pH. The pK_a values for dissociation of the protonated conjugates of the radical-anions are marked by the vertical arrows (see text for sources of data).
All three radicals protonate with \( pK_a \) values for dissociation of the protonated conjugate which influence the kinetics of radical–radical reaction in the physiological range:

\[
\text{SH}^+ \rightleftharpoons S^- + H^+ \quad (20)
\]

with values of \( pK_{20} = 4.8 \) (superoxide \([103]\)), 6.0 (tirapazamine \([100]\)) and 6.1 (metronidazole \([104,105]\)). The form of the curves for superoxide and tirapazamine radicals arises because \( k_{21a} > k_{21b} \) and \( k_{21c} \) is very low or even zero:

\[
\begin{align*}
\text{SH}^+ + S^- ( + H^+) & \rightarrow \text{SH}_2 + S \\
2\text{SH}^+ & \rightarrow \text{SH}_2 + S \\
2S^- ( + 2H^+) & \rightarrow \text{SH}_2 + S
\end{align*} \quad (21a) \quad (21b) \quad (21c)
\]

In the case of metronidazole, the radical has two \( pK_a \) values, one of which probably represents protonation of the unsubstituted imidazole nitrogen (6.1 in the radical, \( \sim 2.5 \) in the ground state \([105]\)). The other:

\[
(\text{SH}_2^2)^+ \rightleftharpoons \text{SH}^+ + H^+ \quad (22)
\]

has a \( pK_{22} = 2.3 \) \([104]\) and we ascribe this to protonation of oxygen in the nitro group in the radical. This latter equilibrium has no influence on \( k_{19} \) in the physiological pH range.

Quinone radical stability is not illustrated on Fig. 5 because it is not possible to generalize and the effects of pH are less marked. The radical chemistry of quinones has been reviewed \([4-6,106,107]\). Simpler \( p- \) and \( o- \)quinone radicals have \( pK_{20} \) values around 4–5 and hence the radicals are dissociated (anions) at pH \( \sim 7 \). For the \( p- \)benzoquinone radical, values of \( k_{19} \sim 2 \times 10^6 \) and \( 1 \times 10^9 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \) at neutral pH and pH 2 were reported \([108]\). For diazaquone (AZQ, Fig. 2, 11), \( 2k_{19} \sim 9 \times 10^6 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \) at pH 7 was measured, 2–3 orders of magnitude lower than for simpler quinone radicals \([109]\).

**First-order (concentration-independent) radical decay pathways**

Not all nitroarene radicals decay according to rate laws defined by disproportionation, eqns. (8) and (19). The radicals of misonidazole (Fig. 2; 2) and other \( 2- \)nitroimidazoles were found to decay exponentially at low concentrations \([110]\). Since radical steady-state concentrations are likely to be submicromolar in cellular systems (except perhaps when totally hypoxic), this is an important decay pathway:

\[
S^-/\text{SH}^+ ( + H^+) \rightarrow \text{products} \quad (23)
\]

At pH 7.3, estimates of \( k_{23} \sim 10 \) and \( 5 \text{ s}^{-1} \) were made for misonidazole and etanida-

zole \([110]\). The reaction rate also decreased with increasing pH (cf. Fig. 5, metronidazole) between pH values of 6.6 and 8.9, with an order of magnitude increase in stability per pH unit. The half-life of reaction (23) (\( \sim 0.7/k_{23} \)) is about 70 ms for misonidazole at pH 7.3.

The final products of reaction (23) may be the same as disproportionation (8): the important point is that the kinetics are independent of radical concentra-
tion. In contrast, the half-lives of 5-nitroimidazole radicals (e.g. metronidazole) were accurately inversely proportional to radical concentration, as expected for a true second-order reaction [eqn. (8)] [110].

Another important group of compounds where unimolecular decay of the radical occurs is nitroarenes substituted with good leaving groups: nitrobenzyl halides are typical. Fig. 6 shows reaction schemes (a) by Teicher and Sartorelli [111] and (b) from the studies of Neta and colleagues [112–115]. In the example from the latter work which is illustrated (dehalogenation of o-nitrobenzyl chloride radical-anion), the formation of a carbon-centred radical occurs with a half-life of \( \sim 60 \mu s \) at 37 °C; the rates vary according to structure and leaving group. From Fig. 4 we expect these short-lived nitro radical-anions to react with oxygen with rate constants, \( k_3 \), no greater than about \( 10^7 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \). The half-life of this 'protective' electron transfer to oxygen \( \{ \sim 0.7/(k_3[O_2]) \} \) will thus be at least \( \sim 2 \text{ ms} \) in well-oxygenated tissue with \([O_2] \sim 40 \mu \text{mol} \cdot \text{dm}^{-3} \). Hence there will be many instances in this group of compounds where peroxy radicals, rather than alkylating reduced nitro compounds, are formed (the carbon-centred radical will react very rapidly with \( O_2 \)). The leaving group will often, but not always, be lost before reaction of nitro radical-anions with oxygen (or with themselves) occurs. These competing pathways are consistent with EPR observations [116].

Shown as an inset to Fig. 6 is an example from recent work [117] which seeks to exploit this chemistry to release a mustard derivative rather than rely on reactive nitroreduction intermediates. It thus seems possible to overcome the kinetic constraints of simpler nitrobenzyl derivatives.

(a) Teicher and Sartorelli (1980) proposed drugs based on:

(b) Pulse radiolysis studies by Neta et al. (1980-1986) showed:

Fig. 6. (a) Hypothetical basis for alkylating DNA following reduction of a nitrobenzyl halide; (b) radical chemistry of o-nitrobenzyl chloride (half-life shown for 37 °C). Inset: compound being evaluated as a bioreductive drug, relying on 'leaving group' chemistry of the radical-anion (see text for references).
Ketyl character

The radical of 4-nitroacetophenone has \(2k_1 \approx 10^7 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}\) invariant with pH for \(\text{pH} > 6\) [3], presumably because some unpaired spin is delocalized on to the ketone function, and the radical has then partial ketyl character. Hence a nitro radical can be made to disproportionate much faster by substitution of an acetyl or similar function. This could be an important consideration in drug design (see below).

Reaction of the initial radical with a target as the mode of action

Evidence for radicals as damaging species

The possible involvement of 'secondary' radicals in drug metabolism [subsequent to the initial formation of \(\text{S}^-\) in eqn. (1)] has been noted above. An illustration is nitroxide radicals formed upon reduction of nitrosoarenes [39]. These radicals may be damaging. Mention has also been made of electrochemical studies indicating interaction of the primary radical-anions, or protonated conjugates, with DNA. However, the simplest evidence for a mechanism of cytotoxicity involving the initial radical as a damaging species arises when the stable 2- and 4-electron reduction products are non-toxic, particularly if reduction stops essentially at the 2-electron step. Then the drug radical, \(\text{S}^-/\text{SH}^+\) is a prime candidate for the toxic intermediate, reaction (6).

This appears to be the case for the di-N-oxide, tirapazamine (Fig. 2; 14) [11,70]. Fig. 7 outlines the reduction chemistry of tirapazamine and a possible basis for its action [70,100,118]. The damage leading to cytotoxicity has been identified as DNA double-strand breaks which are particularly difficult to repair, perhaps comparable with the clustered damage from densely ionizing radiation [119]. Models for the mechanism of action are discussed below.

Fig. 7. Reduction chemistry of tirapazamine and possible mode of action.
Clustered damage from a single radical

This could arise from a reductase present in the nucleus ‘concentrating’ radical attack on DNA [119]. However, we are testing an alternative, novel hypothesis. This involves a single radical initiating a chain reaction which results in multiple strand breaks: (i) a reductase produces a radical, possibly but not necessarily, in the nucleus; (ii) the radical abstracts H from a sugar residue on DNA to give a strand break, much like 'OH radicals but probably several orders of magnitude slower; (iii) a radical centre remains on the sugar which has reducing properties and produces another drug radical from unreduced tirapazamine; (iv) this radical attacks DNA near the site of generation to give a second strand break and damage which is much more clustered than, for example, that from hydrogen peroxide/iron(II).

Although this latter hypothesis is speculative, experimental evidence using a very simple model system suggests further work to explore the possibility is justified. Fig. 8 shows our measurements of the initial rate of loss of tirapazamine obtained when tirapazamine radicals are generated at a constant rate of 55 nmol·dm$\text{-}^{-3}$·s$^{-1}$ by radiolysis of an N$_2$-saturated solution containing tirapazamine (0.5 mmol·dm$^{-3}$) and deoxyribose (50 mmol·dm$^{-3}$). Tirapazamine radicals are produced by reaction of hydrated electrons with tirapazamine and also via 'OH radicals reacting with deoxyribose to produce a reducing radical. The concentration differential ensures no direct reaction of 'OH with tirapazamine.

The radical yield was calibrated by irradiating a similar solution but replacing tirapazamine with ferricyanide and measuring conversion into ferrocyanide; the expected yield was obtained. If there were no chain reaction, then one might expect tirapazamine to be lost by reduction to the 2-electron product at the rate of 0.5 molecules of product per radical input. However, as Fig. 8 shows, the loss was well above 0.5 molecules/radical, and at low pH was some 5-fold higher than expected. The pH dependence is probably associated with protonation of the tirapazamine radical ($pK_a = 6.0$ [100]). We suggest that the results point to a chain reaction in which the propagation step is abstraction of H from the sugar to give

![Fig. 8. Initial rate of loss of tirapazamine upon radiolytic reduction in the presence of deoxyribose.](image-url)
a radical which reduces tirapazamine; the termination steps are radical–radical reactions. The protonated radical evidently abstracts H much faster than the anion-radical. The chain length varies with pH, deoxyribose concentration and dose rate (radical input rate), as would be expected from this model. Qualitatively similar results were obtained with either 2-propanol or formate replacing deoxyribose as H-donor. In addition, preliminary pulse-radiolysis experiments have provided supporting evidence for the model. Full details will be reported elsewhere, but even such a simple model system provides a method of comparing the H-abstracting abilities of radicals from analogues of tirapazamine, to which we plan to extend these studies.

An unusual dependence of tirapazamine-associated cytotoxicity upon oxygen concentration has been reported [120]. This cannot be explained simply on the basis of reactions (3) and (19) competing with reaction (6). If our hypothesis is correct, however, oxygen will also compete by reacting with sugar radicals in an addition/elimination reaction, and the involvement of peroxyl radicals is a possibility. The kinetics of oxygen interaction in our model of tirapazamine reduction involving sugar radicals will be discussed in more detail elsewhere, when pulse radiolysis measurements of such possible competing reactions are complete.

**Implications of the rate constants for competing reactions**

**A model in which the radical reacts with oxygen in competition with disproportionation resulting in toxicity**

In this model, radicals are produced in reaction (1) and can react with oxygen in reaction (3). In competition, radicals can react by disproportionation, reaction (19) [cf. reaction (8)], a pathway which leads to toxicity. By setting the expression for the rate of change of radical concentration with time, \(-d[S^\cdot]/dt = 0\) (the steady-state approximation), it is easily shown that when the rate of reaction (3) equals the rate of reaction (19):

\[ [O_2] = (k_4k_{10}[S])^{1/2}/k_3 \]  

(24)

This will be the oxygen concentration when the toxic pathway is balanced by the non-toxic pathway, i.e. at which the 'switch-over' from oxic protection to anoxic cytotoxicity occurs (radiobiologists sometimes call this the 'K value'). In this expression \(k_4\) is a first-order rate constant (zero-order formalism could have been used, omitting \([S]\)). So far as the use of bioreductive drugs in radiotherapy goes, the oxygen concentration of the critical radioresistant cells is of the order of a few micromolar. Few human tumour cells exist at submicromolar oxygen concentrations [121].

To evaluate this expression, the rate of drug reduction (or radical formation) needs to be measured. This can be readily achieved, e.g. with cells in suspension, measuring loss of drug by HPLC, or measuring oxygen consumption reflecting redox cycling, eqns. (3),(4), etc. However, the measurements indicate radicals produced per cell per unit time, and to derive \(k_4\) we need an estimate of the reaction volume. Siim et al. [21] have argued that the chemistry should be integrated over the total volume of cell suspension, around 1000-fold higher than
the cellular volume in typical experiments. With this assumption, their measured oxygen sensitivity for toxicity of a nitroquinoline agreed with eqn. (24). In support of this assumption, EPR evidence for nitro radicals outside the cellular space in hepatocytes was noted [122]. However, in these latter experiments the cultures would have become anoxic by the time of measurement, and we question whether the assumption of radicals distributed homogeneously throughout the experimental system is valid in the presence of even submicromolar oxygen concentrations. We must consider the question of mean diffusion distance of the radicals.

What is the reaction volume?

It can be shown that the mean diffusion distance, $x$, of a radical in time $t$ is:

$$x = (2Dt)^{1/2}$$

(25)

where $D$ is the diffusion coefficient. For a typical radical in water this will be about $10^{-9}$ m$^2$·s$^{-1}$, but the cytoplasm is more viscous [122], and a value of about $1.5 \times 10^{-10}$ m$^2$·s$^{-1}$ is probably appropriate. The mean lifetime of the radical will depend on oxygen concentration, reductase activity and appropriate rate constants, but if we focus on the important, partially hypoxic range and take the lifetime as about half that of the ‘oxic decay’ pathway, i.e.

$$t \approx (2k_3[O_2])^{-1}$$

then we can estimate the (very approximate) mean radical diffusion distance:

$$x \approx \frac{D}{(k_3[O_2])}^{1/2} \approx \frac{12}{(k_3[O_2])}^{1/2} \text{ nm}$$

(27)

For the nitroquinoline studied by Siim et al. [21], $k_3 \sim 2.5 \times 10^6$ dm$^3$·mol$^{-1}$·s$^{-1}$ and even if $[O_2]$ is as low as 0.5 μmol·dm$^{-3}$, then the mean diffusion distance is only about 11 μm, i.e. of cellular dimensions. This approach ignores the time taken for an anion to permeate the cytoplasmic membrane. We conclude that most of the chemistry we are considering occurs inside the cell except in essentially anoxic cultures. (Diffusion processes should obviously be included in our model of a radical exerting clustered damage which is discussed above; too low a radical reactivity with DNA might permit diffusion away from the target. However, non-homogeneous kinetics are outside the scope of this article.)

The diffusion distance of an intermediate such as a nitrosoarene may also be estimated using the same approach. Since the rate constants for reactions of nitroarene radicals with oxygen are around 1000-fold higher than those for reactions of nitroso intermediates with glutathione, but the concentration of oxygen in hypoxic cells is around 1000-fold lower than glutathione, then the above conclusion concerning the diffusion distances of nitro radicals will also apply, very broadly, to nitrosoarene intermediates produced in cells.

A model in which the radical reacts with oxygen in competition with a first-order pathway resulting in toxicity

We noted above examples, such as 2-nitroimidazoles, where radicals decay by first-order (concentration-independent) pathways, e.g. eqn. (23). If this occurs in competition with reaction with oxygen, eqn. (3), then we do not need to know the
steady-state radical concentration or the reaction volume to estimate the oxygen concentration at which the 'oxic' pathway (3) is balanced by the 'anoxic' pathway (23). When the rates of these two reactions are equal:

$$[O_2] = k_{23}/k_3$$

(28)

and if, for example, $k_{23} \approx 10 \text{ s}^{-1}$ and $k_3 \approx 5 \times 10^6 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ (typical of a 2-nitroimidazole), we might expect a 'K value' [when eqns. (3) and (23) are balanced] of around 2 $\mu$mol·dm$^{-3}$ O$_2$.

**Implications of first-order versus second-order 'anoxic pathways'**

The key difference between the two simple models outlined above is that the first-order 'anoxic pathway' implies the oxygen sensitivity of bioreduction is independent of reduction rate, eqn. (28), in contrast to the consequence of a radical–radical reaction being involved and reduction rate being important in influencing steady-state concentrations of radicals and hence the rate of reaction (19), eqn. (24).

Rauth et al. [123] measured the oxygen inhibition of reduction of a 5-nitroimidazole, metronidazole (the radical of which decays by a second-order pathway), or a 2-nitroimidazole, misonidazole (first-order decay) (see [110]). Reduction involved radiolytic radical generation at a rate of $\sim 10 \text{ nmol} \cdot \text{dm}^{-3} \cdot \text{s}^{-1}$ ($= k_1[S]$). Using the known rate data, $k_3, k_{19}, k_{23}$ for the two compounds [99,110] and eqns. (24) and (28) we can estimate that oxygen should significantly inhibit reduction of metronidazole at a concentration as low as a few nanomolar with the reduction rate used here, but in contrast, inhibiting misonidazole reduction should require $\sim 1000$-fold higher concentrations of oxygen. Experimental results were broadly in line with expectation [123].

Siim et al. [21] found reduction of a nitroquinoline by mammalian cells in suspension to be inhibited at oxygen tensions of about 0.2 $\mu$mol·dm$^{-3}$. The majority of radioresistant, hypoxic cells in tumours exist at oxygen tensions an order of magnitude higher than this value [121,124]. We have questioned above their assumption in their theoretical treatment that the radicals diffuse throughout the experimental volume, but this high sensitivity to oxygen is characteristic of a nitroarene bioreductive drug exhibiting second-order radical–radical decay, eqn. (19). Although a sweeping generalization should be treated with caution, in general it appears that such compounds might be less useful in cancer therapy than, for example, bioreductive drugs based on the 2-nitroimidazole moiety, such as RSU 1069 or its pro-drug, RBU 6145 [125], because of the kinetic factors which distinguish these groups of compounds.

Many quinone radicals disproportionate up to four orders of magnitude faster than nitroarene radicals, but the oxygen sensitivity in eqn. (24) is dependent on the square root of this rate constant, and the difference between the two classes of compound is offset by the $\sim 100$ times higher rate constants for reaction of quinone radicals with oxygen compared with nitro radicals (Fig. 4). Hence the kinetic basis for the use of nitroarene or quinone compounds as bioreductive drugs in cancer therapy has evident parallels even though the absolute rate constants for individual reactions may differ by orders of magnitude.
Conclusions

Bioreductive drugs rely on free radical reactions for their activation and selectivity. Each step in the process can be investigated in model systems. Although the details have not been discussed here, radiolysis methods [101,126] have played a pivotal role in the quantitative understanding of the kinetic factors which control drug efficacy. Redox properties control drug activation and the rates of 'protective' electron transfer to oxygen; pH is important in controlling the rate of radical decay, whether this involves radical–radical reactions or unimolecular processes rate-determining in the decay of some nitroarenes. The effects of pH reflect prototropic equilibria involving radicals, readily quantified by radiolysis methods [100,104,105]. Substituents in aromatic structures will influence both redox properties and radical pKₐ. Thus an electron-donating substituent will generally decrease reduction potential [95] and increase radical pKₐ. In eqn. (24), k₁ will be reduced and k₁₉ could well be increased by such a substituent. In the tirapazamine model described above, a protonated radical is more reactive than the radical-anion, and hence a high radical pKₐ may increase the effectiveness of radical damage at pH > pKₐ. The sensitivity of these parameters to substituent effects could form the basis for rational drug design.

We conclude that reaction kinetics are of central importance in considering the mechanisms of action of bioreductive drugs, and that further work to develop the framework outlined here is desirable. A fuller description of numerical models of the reaction kinetics and oxygen sensitivities of bioreductive drug cytotoxicity will be presented elsewhere.

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References

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