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Pulse radiolysis studies on the hypoxia-selective toxicity of a colbalt – mustard complex

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Summary The kinetic basis for the *in vitro* hypoxia-selective cytotoxicity (HSC) of the Co(III)-nitrogen mustard complex SN 24771 (NSC 675352) has been investigated using pulse radiolysis. The rate constants for the one-electron reduction of SN 24771 by model reductants exhibited a marked dependence on the one-electron reduction potential of the reductant, with values up to several orders of magnitude slower than for the nitroimidazole drug misonidazole. Following one-electron reduction to form the Co(II) complex (species I) consecutive conversion to further transient species (II and III) occurs with first order rate constants of 120 ± 10 s^{-1} and 10 ± 2 s^{-1} and are associated with release of ligands. Neither of these subsequent processes are inhibited by the addition of O_2 up to a concentration of 0.5 mmol l^{-1} suggesting that the HSC action of SN 24771 most likely arises from a mechanism other than simple redox cycling between the Co(III) and Co(II) forms by O_2. If the measured low rate constants of one-electron reduction by model reductants of SN 24771 (as compared to the reduction of nitroaromatics), is mirrored by biological reductants, then it is proposed that HSC may occur through competition between SN 24771 and O_2 for these reductants.

Keywords: bioreductive drug; hypoxia-selective cytotoxicity; one-electron reduction

The cobalt(III)-nitrogen mustard complex SN 24771 (NSC 675352; $[Co(III)(Meacac)_2(DCE)]^+$ where DCE is *N,N*-bis(2-chloroethyl)ethylenediamine and $Meacac^-$ is 3-methyl-2,4-pentanedionato anion) is a bioreducible metal complex with hypoxia-selective cytotoxicity (HSC) to cells *in vitro*, through release of the cytotoxic mustard, DCE (Ware *et al.*, 1993; Wilson *et al.*, 1994). Part of the interest in such metal complexes stems from the fact that, unlike many organic bioreductive drugs which can undergo both initial one-electron reduction (oxygen sensitive) and two-electron reduction (usually insensitive to oxygen), for the cobalt (III) complexes only one-electron reduction is possible. It has been suggested that HSC of SN 24771 arises in an analogous manner to nitroaromatic compounds (scheme 1) with back-oxidation of the initial one-electron adduct by oxygen inhibiting metabolic formation of a cytotoxin (Wilson, 1993; Denny and Wilson, 1993). The mechanism of cytotoxicity of SN 24771 appears to be the same under aerobic and hypoxic conditions, suggesting that release of DCE is not completely suppressed by O_2 (Ware *et al.*, 1993). The kinetics of reduction and the competition between reoxidation by O_2 and cytotoxic ligand release from the intermediate (species I) of this class of HSC has not been previously studied. Rational development of more selective compounds requires a quantitative understanding of these processes. The fast kinetic spectrophotometry technique of pulse radiolysis is ideally suited for this purpose and is able to give insights into the HSC mechanism of cobalt(III)-nitrogen mustard complexes.

Materials and methods

SN 24771 was synthesised in this laboratory and purified by ion exchange chromatography followed by recrystallisation as the perchlorate salt (Ware *et al.*, 1993). Purity was determined by reverse-phase high-performance liquid chromatograpy (HPLC) (>98%). $[Co(II)(acac)_2]$ ($acac^-$ = 2,4-pentanedionato anion) was prepared from $[Co(II)(acac)_2(H_2O)_2]$ by standard methods (Ellern and Ragsdale, 1968). [Co(II)(acac)$_2$(DEE)] (DEE = *N,N*-bis(ethyl)ethylenediamine) was prepared *in situ* by the addition of an excess of DEE to a solution of $[Co(II)(acac)_2]$ in CH_2Cl_2.

Pulse radiolysis was performed using the new facility at The University of Auckland, consisting of a modified Dynaray 4 linear accelerator (200 ns pulse length, typical radiation dose of 6 Gy), optical detection and PC-controlled data harvesting and analysis. Solutions for radiolysis were either purged free of O_2 using N_2O gas, or controlled amounts of O_2 were added by purging with mixtures of O_2/N_2O using calibrated flow meters. Time-resolved spectra of the radical species produced following one-electron reduction of SN 24771 are presented as the product of the radiation chemical yield (G, μmol J^{-1}) and change in extinction coefficient relative to the absorbance of the unreduced parent compound ($\Delta\varepsilon$, 1 mol^{-1} cm^{-1}). (For a full radical yield of 0.6 μmol J^{-1} a $G\Delta\varepsilon$ of 0.006 corresponds to a $\Delta\varepsilon$ of 10 000 1 mol^{-1} cm^{-1}.) Steady-state radiolysis were carried out using a ^{60}Co γ-ray source (dose rate 52 Gy min^{-1}). Cumulative doses were given to solutions contained in an anaerobic vessel equipped with an attached quartz cell for uv/vis spectrophotometry.

Results

One-electron reduction of SN 24771

Reduction of SN 24771 through electron transfer from both the $CO_2^{\bullet -}$ species and the well characterised one-electron reduced form of methylviologen [$E(1) = -447$ mV] was too slow ($c. \leqslant 10^6$ 1 mol^{-1} s^{-1}) to permit the observation of fast unimolecular events. Fast one-electron reduction of SN 24771 (0.5 mmol l^{-1}) was achieved in N_2O-saturated aqueous solution containing sodium formate (0.15 mol l^{-1}) and electron mediators of low E(1) such as certain viologens (V^{++}, -0.74 V to -0.64 V) or *N*-methylnicotinamide (MeN^+, min 1.01 V) (5 mmol l^{-1}). The following reactions are completed within a few microseconds giving rise to the one-electron reduced species of SN 24771, the Co(II) complex, species I.

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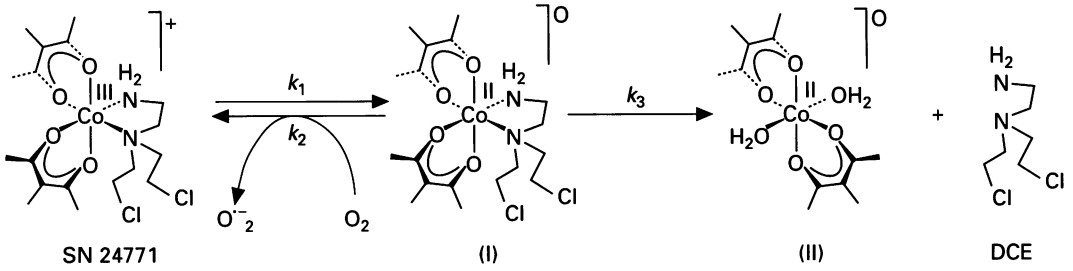


Scheme 1.

$$H_2O \wwwrightarrow {}^{\bullet}OH, H^{\bullet}, e^-_{aq}, H_2, H_2O_2, H_3O^+$$
$$e^-_{aq} + N_2O \rightarrow {}^{\bullet}OH + OH^- + N_2$$
$${}^{\bullet}OH(H^{\bullet}) + HCOO^- \rightarrow CO_2^{\bullet -} + H_2O(H_2)$$
$$CO_2^{\bullet -} + MeN^+(V^{+\,+}) \rightarrow MeN^{\bullet}(V^{+\bullet})$$

$$MeN^{\bullet}(V^{+\bullet}) + [Co(III)(Meacac)_2(DCE)]^+ \rightarrow MeN^+(V^{+\,+}) + [Co(II)(Meacac)_2(DCE)] \quad (I)$$

Rate constants for the reduction of the Co(III) centre of SN 24771, monitored both by following the decrease in absorption of the reductants and by the formation of the transient species I, exhibited a strong dependence on the one-electron reduction potential [$E(1)$] of the reductant (Figure 1). The second order rate constants were determined by measuring the increase in observed rates upon varying the concentration of SN 24771 (0.05–0.3 mmol l^{-1}). The kinetic data obtained are distinctly different from that obtained for nitroaromatic compounds, such as misonidazole, for which the rate constants of reduction by these reductants exhibit little dependence on $E(1)$, all being close to diffusion controlled (Figure 1). Species I is unstable, converting in a sequential manner to two further transient species over the timescale of pulse radiolysis observation (up to 0.5 s) (Figure 2 and Insert A). Species II was formed with a rate constant of 120 ± 10 s^{-1} and in turn decayed to species III with a rate constant of 10 ± 2 s^{-1}. The rate constants for the formation of species II and III are first order, as they are independent of the concentration of SN 24771 (0.1–0.5 mmol l^{-1}) and the radiation dose (i.e. changes in the concentration of reductant radicals and hence the concentration of species I). Species III is not the final product as steady-state radiolysis experiments revealed that the final products do not absorb significantly at any wavelengths above 300 nm, consistent with the formation of $[Co(II)(H_2O)_6]^{2+}$ and the free ligands (Figure 3). Complete loss of parent compound (SN 24771, 85 μmol l^{-1}) is achieved upon stoichiometric one-electron reduction (140 Gy produces 84 μmol l^{-1} of reducing MeN$^{\bullet}$ radicals).

Reaction of intermediate species with oxygen

Use of mixtures of O_2/N_2O up to $[O_2]$ = 0.5 mmol l^{-1} lowered the yield of species I (and hence the yields of II and III also) due to competition by O_2 for the electron mediators, but had no detectable affect on the rate constants for the formation of species II and III. (Figure 2, Insert trace C). Species I was, however, oxidised by benzoquinone, (Figure 2, Insert trace B) with $k = 0.9 \times 10^5$ l mol^{-1} s^{-1} (this rate constant is determined after subtraction of the kinetic component due to the conversion of species I to II), consistent with the regeneration of the parent compound in competition with the formation of species II.

Comparative spectra

The uv/vis spectra of the complexes $[Co(II)(acac)_2(H_2O)_2]$ and $[Co(II)(acac)_2(DEE)]$ were measured in dry CH_2Cl_2 in which the integrity of these labile complexes is greatly enhanced. In the region of interest (300 to 440 nm) the spectra are quite similar, with $[Co(II)(acac)_2(DEE)]$ absorbing slightly more than $[Co(II)(acac)_2(H_2O)_2]$, comparable with the difference observed between species I and II (Figure 2).

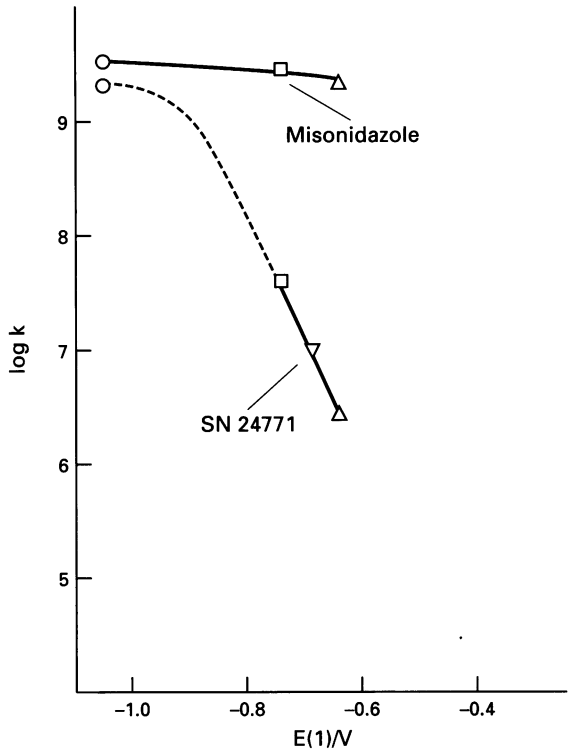


Figure 1 Dependence of the rate constant (k) of electron transfer to SN 24771 and misonidazole on the one-electron reduction potential, $E(1)$ of the reductant. Reductants [with $E(1)$ values] are the one-electron reduced forms of MeN^+(−1.01V), ○; $V41^{2+}$(−0.74 V),□;$V31^{2+}$(−0.69V),▽;TeQ^{2+}(−0.64V),△; as described in Anderson (1976) and Anderson and Patel (1984).

Discussion

Sequential loss of bidentate ethylenediamine ligands (en) from $[Co(II)(en)_3]^{2+}$, formed by pulse radiolysis of $[Co(III)(en)_3]^{3+}$, has been observed in aqueous solution using conductivity detection (Lilie *et al.*, 1976; Shinohara *et al.*, 1977). These studies were carried out over a limited pH range (3–4.5) and showed that the rate of loss of each ligand decreased with decreasing $[H_3O^+]$. At pH 4.5 the rate constant for the loss of the first en ligand from the transient $[Co(II)(en)_3]^{2+}$ complex is *c.* 350 s^{-1}, which is consistent with the observed kinetics of loss of the first ligand from species I in the present study (120 ± 10 s^{-1}) taking into account differences in pH, charge and structure between the two systems. This observation, along with the similarity in absorption spectra of $[Co(II)(acac)_2(H_2O)_2]$ and $[Co(II)(acac)_2(DEE)]$ suggests that the DCE ligand of SN 24771 is released first followed sequentially by the two $Meacac^-$ ligands which cause the observed major changes in the uv/vis spectrum.

Loss of the acetylacetonate ligands ($acac^-$) from $[Co(II)(acac)_3]^-$ has also been studied (Meisel *et al.*, 1979). Sequential replacement of $acac^-$ by H_2O occurs with rate constants $8 \pm 2 \times 10^3$ s^{-1}, 30 ± 10 s^{-1} and 3 ± 1 s^{-1}, and transient species $[Co(II)(acac)_2(H_2O)_2]$ and $[Co(II)(acac)(H_2O)_4]^+$ are formed. The rate constant for loss

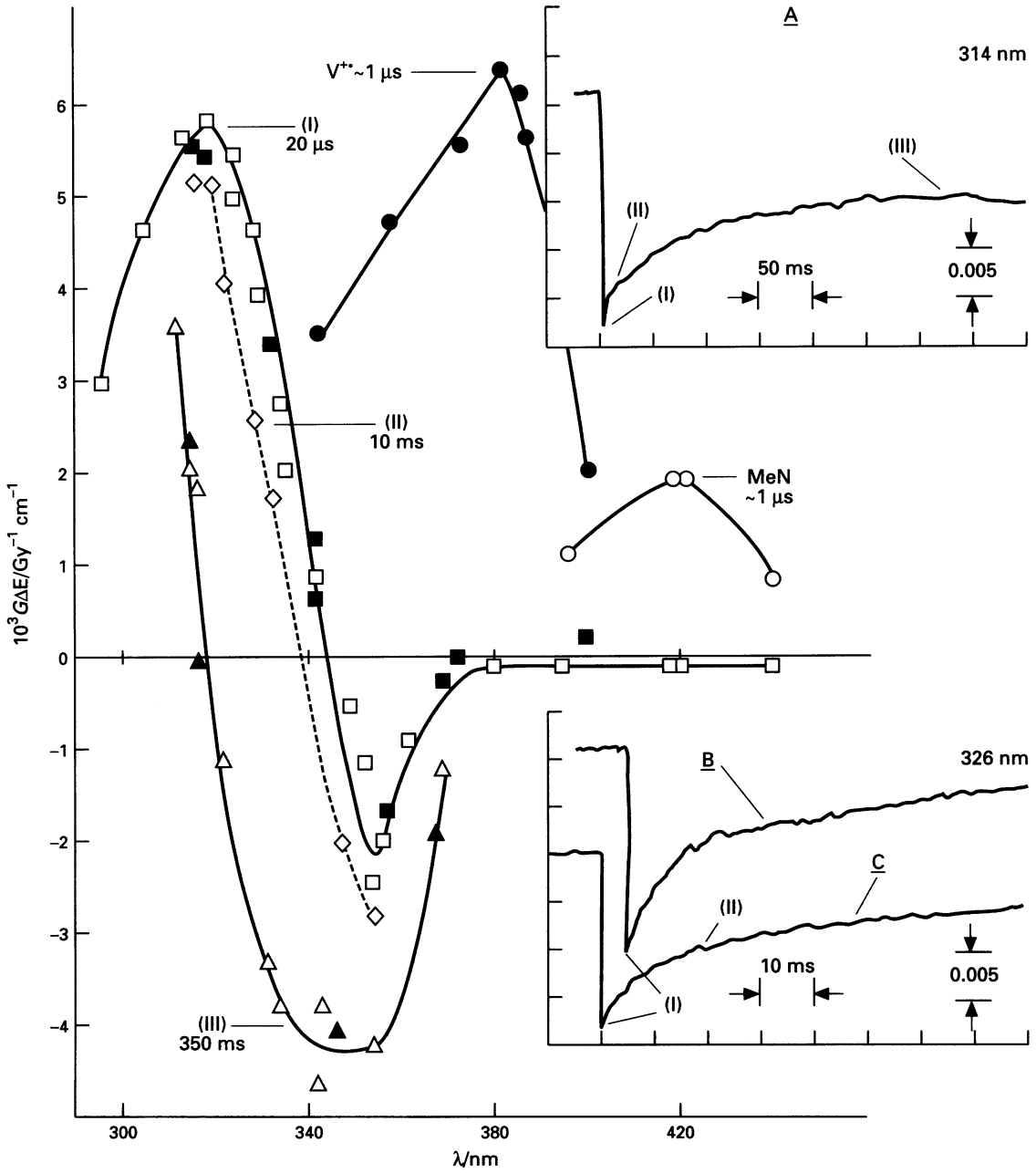


Figure 2 Time-resolved transient spectra formed following pulse radiolysis (6.5 Gy in 200 ns) of solution described in Results. Changes in absorption are relative to parent SN 24771. Open symbols are events initiated by $MeN^{\bullet}$ radicals, closed symbols by $V41^{+\bullet}$ radicals. Inserts are oscillograms of optical density vs time upon reaction of $MeN^{\bullet}$ radicals with SN 24771. *Trace A*, in the absence of O_2; *Trace B*, in the presence of benzoquinone (100 μmol l^{-1}) at a radiation dose of 3 Gy (Data are fitted by two consecutive first order reactions with k_1 = 203 ± 7 s^{-1} and k_2 = 10 s^{-1}); and *Trace C*, in the presence of O_2 (0.5 mmol l^{-1}) at a radiation dose of 7.5 Gy. (Data are fitted by two consecutive first order reactions with k_1 = 116 ± 9 s^{-1} and k_2 = 9 s^{-1}). Fitted curves are omitted for clarity.

of $acac^-$ from $[Co(II)(acac)_2(H_2O)_2]$ is only slightly larger than we observe for conversion of transient species II to III. We have confirmed this assignment (RF Anderson and DC Ware, unpublished work) by use of $Co(III)(acac)_2DCE$, which showed the same kinetics for loss of the $acac^-$ ligand as previously reported from $[Co(II)(acac)_2(H_2O)_2]$ (Meisel *et al.*, (1979).

The above considerations support the following proposed sequence of reactions:

$[Co(II)(Meacac)_2(DCE)]$ [I]→
$[Co(II)(Meacac)_2(H_2O)_2]$ (II) + DCE ($k = 110 \pm 10$ s^{-1})

$[Co(II)(Meacac)_2(H_2O)_2]$ [II]→
$[Co(II)(Meacac)(H_2O)_4]^+$ (III) + $Meacac^-$ ($k = 10 \pm 2$ s^{-1})

$[Co(II)(Meacac)(H_2O)_4]^+$ [III]→
$[Co(II)(H_2O)_6]^{2+}$ (final product) + $Meacac^-$ ($k << 10$ s^{-1})

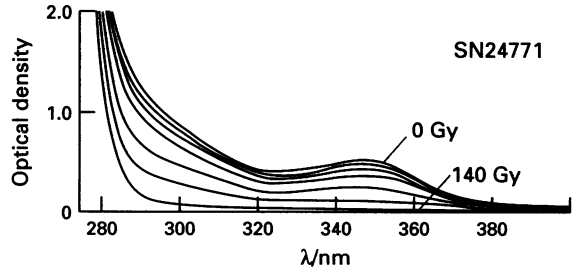
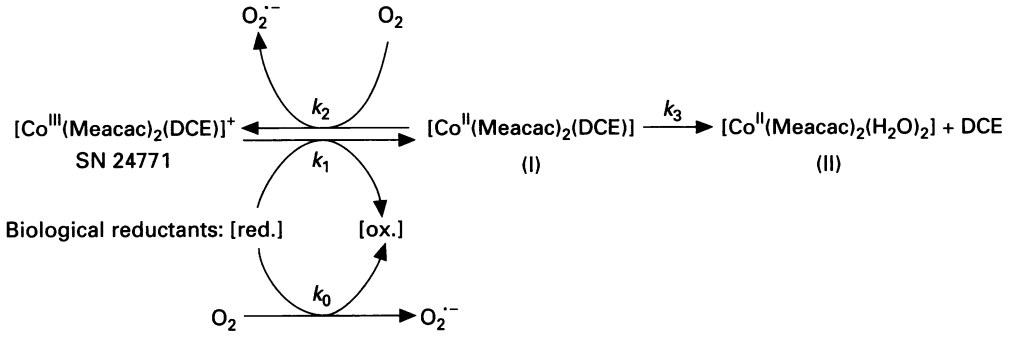


Figure 3 Spectral changes following steady-state γ-radiolysis of SN 24771 (85 μmol l^{-1}) in a N_2O-saturated solution containing sodium formate (0.1 mol l^{-1}) and *N*-methylnicotinamide (2 mmol l^{-1}) buffered at pH 7 (phosphate, 5 mmol l^{-1}). Radiation doses are 10, 20, 40, 80, 120 and 140 Gy. (Absorption below 300 nm due to *N*-methylnicotinamide in addition to SN 24771).



Scheme 2

Loss of each ligand would be expected to raise the redox potential of the complex, making oxidation of the Co(II) centre by O_2 progressively more unfavourable. If the redox dependency (c. an order of magnitude change in the rate constant for electron transfer per ΔE of 100 mV) and other electron transfer parameters are similar for the oxidation of the Co(II) species (I) and the reduction of the parent Co(III) complex, then based on the rate constant measurement using benzoquinone and the recalculated $E(1)$ of benzoquinone being +78 mV (Wardman, 1989), the predicted rate constant for the oxidation of species I by O_2 ($E(1) = -155$ mV (Meisel and Czapski, 1975)) is *c.* 10^4 l mol^{-1} s^{-1}. This estimated rate constant is consistent with the lack of effect of O_2 on the fate of species I in the present study, and indicates that under aerated conditions ($[O_2]$ = c. 260 μmol l^{-1}) O_2 will not back-oxidise species I to a significant extent in competition with release of ligands.

The slow kinetics of reoxidation of the initial SN 24771 reduction intermediate by O_2 strongly suggests that the HSC action of this metal complex does not result from one-electron redox cycling as originally proposed (scheme 1). In this respect SN 24771 appears to be distinctly different from the majority of organic bioreductive drugs, for which one-electron redox cycling appears to be the major mechanism by which O_2 inhibits metabolic reduction. If the low rate constants for one-electron reduction of SN 24771 by chemical reductants (as compared with the reduction of nitroaromatics) are representative of biological reductants, then oxygen inhibition may occur through competition between SN 24771 and O_2 for these reductants (scheme 2). HSC may thus arise because $k_0[O_2] > k_1[\text{SN 24771}]$ in cellular systems (scheme 2), rather than because $k_2[O_2] > k_3$. While there is strong evidence that redox cycling is the major mechanism by which O_2 inhibits net reduction of nitroaromatic compounds in cells (Orna and Mason, 1989; Siim and Wilson, 1995), there may be other instances (e.g. reduction of tertiary amine N-oxides, in which kinetic competition for reducing species contributes to the oxygen sensitivity of bioreductive drug activation.

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