Pulse-radiolysis study of daunorubicin redox cycles

Reduction by e^-_{aq} and $COO^{\cdot -}$ free radicals

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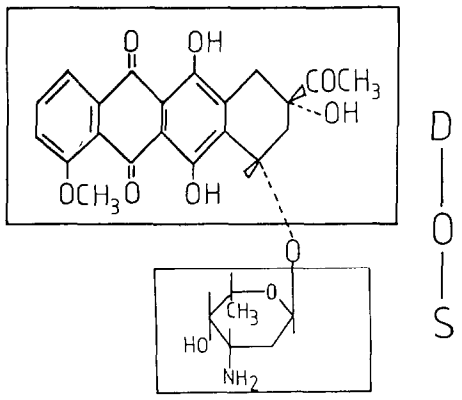
Received 17 October 1984

Daunorubicin aqueous solutions were reduced by COO^- or e^-_{aq} free radicals produced by pulse radiolysis. The kinetics of the semiquinone radical formation and decay were studied. The semiquinone disproportionation leads to a pseudo-equilibrium between the drug, its semiquinone and hydroquinone reduced states (K_{eq} = 30 at pH 7), which lasts a few hunderd milliseconds and which is destroyed by the hydroquinone glycosidic cleavage. Consequences concerning daunorubicin antitumour action are briefly discussed.

Daunorubicin *Pulse radiolysis* *Free radical* *Semiquinone* *Hydroquinone* *Redox cycle*

1. INTRODUCTION

Daunorubicin (daunomycin) (DOS) is an anthracycline antitumour antibiotic frequently used in cancer chemotherapy. Its clinical use is limited by cardiotoxicity [1], which seems to be related to free radical formation [2].



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Abbreviations: DOS, daunorubicin; $DOS^{\cdot -}$, semiquinone daunorubicin; DH_2OS, hydroquinone daunorubicin

We have recently investigated daunorubicin reduction by COO^- free radicals produced by γ-radiolysis [3]. The results obtained suggested the formation of the semiquinone as an intermediate, its disproportionation giving an unstable hydroquinone form which loses its sugar moiety.

This study uses the fast kinetics method of pulse radiolysis which enables us to characterize the semiquinone and hydroquinone transients by their absorption spectra and radical decay kinetics.

2. MATERIALS AND METHODS

Daunorubicin–HCl was obtained from Sigma. All chemicals for irradiation (NaH_2PO_4, NaOH, NaCOOH, *t*-butanol) were Rhône-Poulenc 'Normapur'. Water was triply distilled and its purity controlled by conductivity ($\leqslant 10^6\ \Omega^{-1}\cdot cm^{-1}$) or purified through a Millipore Q-system ($18\ M\Omega^{-1}\cdot cm^{-1}$).

The ~800 Gy electron pulses were produced by a Febetron 708 (Paris) (double electron beam 800 keV, 4 ns, optical path 1 cm), the 20–70 Gy electron pulses were produced by a Febetron 707 (Saclay) (single electron beam 1.8 MeV, 8 ns, optical path 2.5 cm). Some experiments (8–50 Gy

Published by Elsevier Science Publishers B.V.
00145793/85/$3.30

pulses) were made using the linear accelerator facility at Ohio State University (USA) (single electron beam ~4 MeV, 0.1–1 μs pulses, optical path 4 cm).

3. EXPERIMENTAL RESULTS

3.1. *Free radical formation*

The reducing species were either $COO^{\cdot -}$ (produced by irradiation of solutions containing sodium formate 0.1 mol·dm^{-3} saturated with N_2O) or e^-_{aq} (produced by irradiation of solutions containing *t*-butanol 0.1 mol·dm^{-3} saturated with N_2 or Ar), or both (produced by irradiation of solutions containing sodium formate 0.1 mol·dm^{-3} saturated with N_2 or Ar). $[DOS]_o$ varied between 2.5×10^{-5} mol·dm^{-3} and 1.77×10^{-4} mol·dm^{-3} and the dose between 8 and 42 Gy (5×10^{-6} mol·dm$^{-3} \leqslant [COO^{\cdot -}]_o \leqslant 2.6 \times 10^{-5}$ mol·dm^{-3}).

Fig.1 shows $DOS^{\cdot -}$ free radical difference absorption spectrum obtained in the following conditions: $[DOS]_o = 5.3 \times 10^{-5}$ mol·dm^{-3}, pH 7 (phosphate buffer 6×10^{-2} mol·dm^{-3}); $[HCOO^-] = 0.1$ mol·dm^{-3}, N_2O, dose ~26 Gy. The spectrum was taken 20 μs after the pulse between 350 and 700 nm where the contribution of $COO^{\cdot -}$ is negligible. This time corresponds to ΔOD maxima at $\lambda \geqslant 600$ nm where DOS does not absorb. During $DOS^{\cdot -}$ formation, this spectrum moves identically in the whole wavelength range. As an example, we show on fig.1 the experimental points obtained 4 μs after the pulse and normalized at 600 nm.

Absorptivities (fig.1, right scale) were determined by slopes of straight lines $\Delta OD^{\lambda}_{max}$ *vs* $[COO^{\cdot -}]_o$ at different wavelengths between 600 and 700 nm under first order conditions (see, for example, ΔOD^{650}_{max} *vs* $[COO^{\cdot -}]_o$, inset B of fig.1).

The spectrum obtained by reaction of the drug with e^-_{aq} was the same. Both are similar to the adriamycin semiquinone spectrum obtained in [4,5].

The $DOS^{\cdot -}$ formation kinetics was studied at $\lambda \geqslant 600$ nm under pseudo-first order conditions, so that reducing radical reaction with itself did not occur in the presence of the drug, and the apparent rate constant, k_{app}, was determined by slopes of first order plottings (fig.2A,B).

$COO^{\cdot -}$ reaction (fig.2A). In daunorubicin solutions, the only reaction of $COO^{\cdot -}$ is:

$$COO^{\cdot -} + DOS \longrightarrow DOS^{\cdot -} + CO_2 \qquad (1)$$

k_1 is equal to the slope of the straight line k_{app} *vs* $[DOS]_o$ of fig.2A:

$$k_1 = (2.0 \pm 0.2) \times 10^9 \text{ mol}^{-1}\cdot\text{dm}^3\cdot\text{s}^{-1}$$

e^-_{aq} reactions (fig.2B). In daunorubicin solutions, the kinetic scheme is:

$$e^-_{aq} \longrightarrow \text{products} \qquad (2)$$

$$e^-_{aq} + DOS \longrightarrow DOS^{\cdot -} \qquad (3)$$

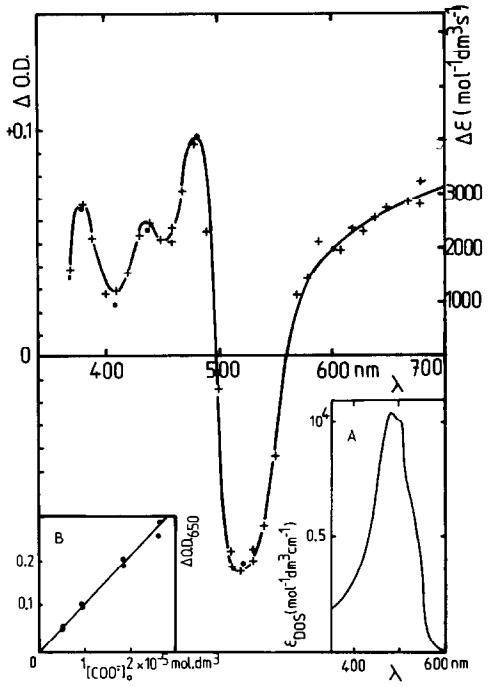

Fig.1. Difference absorption spectrum of the semiquinone radical $DOS^{\cdot -}$ obtained by DOS reduction with $COO^{\cdot -}$. $[DOS]_o = 5.3 \times 10^{-5}$ mol·dm^{-3}, phosphate buffer 6×10^{-2} mol·dm^{-3}, pH 7, sodium formate 10^{-1} mol·dm^{-3} under N_2O, dose 26 Gy, optical path 2.5 cm; (+) 20 μs after the pulse; (●) 4 μs after the pulse, normalized at 600 nm. Right scale: difference molar absorption coefficient of DOS^- radical. Inset A: absolute spectrum of daunorubicin. Inset B: variation of ΔOD^{650}_{max} with $[COO^{\cdot -}]_o$ obtained in pseudo-first order conditions. $[DOS]_o = 1.24 \times 10^{-4}$ mol·dm^{-3}, phosphate buffer 5×10^{-3} mol·dm^{-3}, pH 7, sodium formate 5×10^{-3} mol·dm^{-3}, optical path 4 cm.

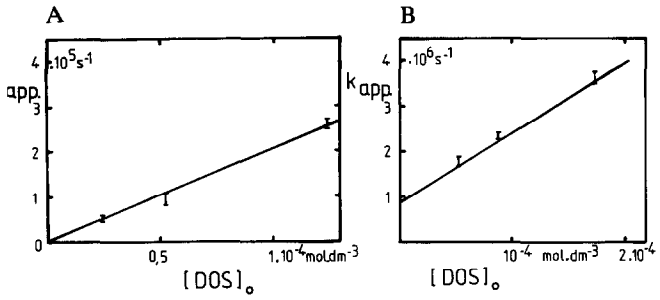

Fig.2. Variation of the slopes of the pseudo-first order plottings k_{app} with [DOS]$_o$.

(A) Reduction by $COO^{\cdot -}$ radicals (N_2O, [$HCOO^-$] = 0.1 mol·dm^{-3})

[DOS]$_o$ (mol·dm^{-3})	Dose (Gy)	[$COO^{\cdot -}$]$_o$ (mol·dm^{-3})
1.24×10^{-4}	8.12	5×10^{-6}
1.24×10^{-4}	14.70	9.2×10^{-6}
5.3×10^{-5}	19	1.18×10^{-5}
2.5×10^{-5}	8.12	5×10^{-6}

(B) Reduction by e^-_{aq} (Ar, *t*-butanol, 0.1 mol·dm^{-3})

[DOS]$_o$ (mol·dm^{-3})	Dose (Gy)	[e^-_{aq}]$_o$
1.77×10^{-4}	115	3.35×10^{-5}
8.85×10^{-5}	72	2.11×10^{-5}

Reaction 2 symbolizes the global reaction of e^-_{aq} with phosphate anions [6]. k_2 was determined by e^-_{aq} decay in the buffer corresponding to a kinetic scheme made of reactions 2 and 4:

$$e^-_{aq} + e^-_{aq} \longrightarrow 2\ OH^- + H_2 \qquad (4)$$

e^-_{aq} disappearance was simulated using an iteration program. This led to:

$k_2 = 7 \times 10^5\ s^{-1}$ (pH 7, [phosphate]$_o$ = 6×10^{-2} mol·dm^{-3})

In daunorubicin solutions, and in the conditions described above, only reaction 2 and 3 occur, and the slope of the first-order plottings k_{app} is equal to:

$$k_{app} = k_2 + k_3\ [DOS]_o$$

The intercept of the straight line k_{app} *vs* [DOS]$_o$ (fig.2B) is equal to $7 \times 10^5\ s^{-1}$ and is in very good agreement with the value found in the buffer. The straight line slope gives:

$$k_3 = (1.63 \pm 0.03) \times 10^{10}\ mol^{-1} \cdot dm^3 \cdot s^{-1}$$

3.2. *Semiquinone free radical decay*

The semiquinone radical decay observed at many wavelengths from 380 to 700 nm was neither a first-order nor a second-order reaction ([DOS]$_o$ between 2.5×10^{-5} mol·dm^{-3} and 1.77×10^{-4} mol·dm^{-3}; doses between 8 and 70 Gy). The difference spectra which were stable for hundreds of milliseconds, such as the one in fig.3, look different from each other, depending on [DOS]$_o$ and on the dose chosen in the range cited above. An analogous phenomenon was found for

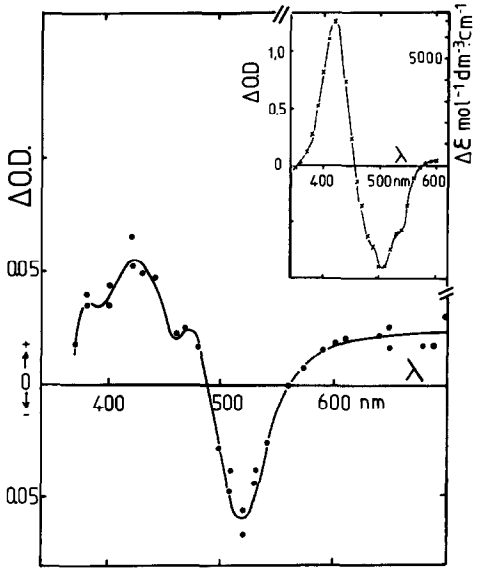

Fig.3. Difference absorption spectrum measured 100 μs after the pulse and stable from 100 μs to 500 ms after the pulse. [DOS]$_o$ = 1.77×10^{-4} mol·dm^{-3}, dose 35 Gy, phosphate buffer 6×10^{-2} mol·dm^{-3}, [HCOONa]$_o$ = 10^{-1} mol·dm^{-3}, under N_2O, optical path 1 cm. Points are experimental and the curve is computed (see section 3.2). Inset: Difference absorption spectrum of DH_2OS hydroquinone obtained by $COO^{\cdot -}$ and e^-_{aq} reduction in solutions containing [DOS]$_o$ = 1.77×10^{-4} mol·dm^{-3}, phosphate buffer 6×10^{-2} mol·dm^{-3}, pH 7, [HCOONa]$_o$ = 10^{-1} mol·dm^{-3}, under N_2, dose 800 Gy, optical path 1 cm. Right scale: difference DH_2OS absorptivities.

naphthazarin semiquinone, a compound which has the same quinone-hydroquinone groups, and was explained by a semiquinone/hydroquinone equilibrium [7]. We therefore suggest a similar kinetics for $DOS^{\cdot -}$ decay:

$$2\ DOS^{\cdot -} \underset{(-5)}{\overset{(5)}{\rightleftharpoons}} DOS + DH_2OS$$

Thus, we propose that the observed spectra (fig.3) should be a sum of $DOS^{\cdot -}$ and DH_2OS absorptions.

The absorption spectrum of hydroquinone compound DH_2OS (fig.3 inset) was obtained by irradiation of a drug solution (1.77×10^{-4} mol·dm^{-3}) with a dose of 800 Gy and measured 100 μs after the pulse. The reducing species were $COO^{\cdot -}$ and e^-_{aq} together. The initial amount of reducing radicals was equal to $\sim 5 \times 10^{-4}$ mol·dm^{-3}. With such an amount of reducers, we assume that all the antibiotic molecules were transformed into their hydroquinone forms by two successive reductions, and hence the extinction coefficients for the hydroquinone were deduced.

The disproportionation kinetics was studied in conditions detailed above (see beginning of section 3.2).

$DOS^{\cdot -}$ decays could be considered as second order reactions for one half-life and $2k_5$ thus estimated:

$$2k_5 = (1.4 \pm 0.4) \times 10^9\ \text{mol}^{-1}\cdot\text{dm}^3\cdot\text{s}^{-1}$$

The equilibrium constant K_{eq} was evaluated at the end of the decays ($t > 500\ \mu$s) using $\Delta\epsilon$ of $DOS^{\cdot -}$ and DH_2OS at the final plateau: $15 < K_{eq} < 100$. These values were checked by simulating $DOS^{\cdot -}$ formation and decay measured between 600 and 700 nm, using an iteration program and a kinetic scheme made up of reactions 1, 5 and -5. The best fit to experimental results was obtained for:

$$2k_5 = (1.2 \pm 0.2) \times 10^9\ \text{mol}^{-1}\cdot\text{dm}^3\cdot\text{s}^{-1}$$

$$k_{-5} = (2 \pm 1) \times 10^7\ \text{mol}^{-1}\cdot\text{dm}^3\cdot\text{s}^{-1}$$

and hence:

$K_{eq} = \dfrac{k_5}{k_{-5}}$ is equal to:

$$14 \leqslant K_{eq} \leqslant 33$$

These values and the equilibrium hypothesis were also checked by computing the experimental difference absorption spectra such as the one shown on fig.3 using $\Delta\epsilon(DOS^{\cdot -})$ (fig.1), $\Delta\epsilon(DH_2OS)$ (fig.3 inset) and the same kinetic scheme.

Fig.3 shows a good agreement between the experimental values and a computed spectrum (solid line), which suggests that the hypotheses, including the total 2-electron reduction of the drug by 800 Gy irradiation, were correctly done.

The standard potentials of redox couples $DOS/DOS^{\cdot -}$ and $DOS^{\cdot -}/DH_2OS$ were calculated from K_{eq}:

$$DOS \quad \underline{-0.43\ V} \quad DOS^{\cdot -} \quad \underline{-0.39\ V} \quad DH_2OS$$

$$-0.41\ V\ [8]$$

4. CONCLUSION

Our aim was to study the semiquinone radical formation and disproportionation initiated by daunomycin reduction by e^-_{aq} or $COO^{\cdot -}$ free radicals. We showed that this semiquinone free radical could be formed by these two different reducing agents, and that its decay led to a pseudo equilibrium which is destroyed by hydroquinone glycosidic cleavage [3]. However, this equilibrium should be taken into account in the mechanism of cytotoxicity of this drug because it gives the semiquinone an abnormally long lifetime. Thus, semiquinones generated in vivo might have enough time to diffuse and to intercalate or to react with important biological targets. This would be consistent with the findings [9] of anthracycline semiquinone binding to DNA.

ACKNOWLEDGEMENTS

We are indebted to B. Hickel and J. Potier for the use of the Febetron 707 and to J. Chevrel for his skilful assistance in the use of the Febetron 708. We wish to thank Mr E. Ray for his assistance in maintaining the LINEAC facility at Ohio State University.

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